Vol. XXXIX October, 1950 No. 10

SCIENTIFIC EDITION

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CONTENTS OF THIS ISSUE

The Decomposition of Equilin in Oil Solution 541
Daniel Banes, Jonas Carol, and Edward O. Haenni

Iron-Kober Reagent: A More Sensitive Stable Reagent for the Determination of *Alpha*- and *Beta*-Estradiols 544
Edward O. Haenni

Toxicity and Repellency to Rats of Actidione 552
Robert Traub, James B. DeWitt, Jack F. Welch, and Doris Newman

The Spectrophotometric Examination and Acute Toxicity of Rutins Obtained from *Fagopyrum esculentum* (Buckwheat) and *Sophora japonica* 556
J. W. E. Harrisson, Bella W. Seltzer, and Eric W. Martin

Experiments with the Pressure Cooker in Drug Extraction 560
Salvatore J. Greco and Andrew G. Duriez

Study of Odors 565
N. N. Tunyolac and J. R. Eaton

A Study of Monoalkylcarbamates as Local Anesthetics 575
D. A. Schlichting, G. E. Cwalina, and G. L. Jenkins

Studies on *Mahonia* Genus. II. Botany and Chemistry of *M. acanthifolia* Don 577
R. Chatterjee and M. P. Guha

A Convenient Laboratory Preparation of Ethylmorphine Hydrochloride, U. S. P. XIII 581
Manuel M. Baizer and Kurt S. Ellner

Toxicological Studies on Synthetic Glycerin 583
Robert C. Anderson, Paul N. Harris, and K. K. Chen

Cultivation Studies of the Solanaceous Drugs. II 586
W. R. Brewer and L. David Hiner

Investigation of *Chrysactinia Mexicana*, A. Gray 591
Lewis Lappas and Carroll B. Gustafson

A Note on the Purification of Dehydrocholic Acid 595
Jose Erdos

A Note on the Isolation of Ursolic Acid from *Verbena stricta*, Vent 595
Nydia M. King, Asima Chatterjee, and Lloyd M. Parks

Book Reviews 596

1951 Iodine Research Award Nominations Requested 598

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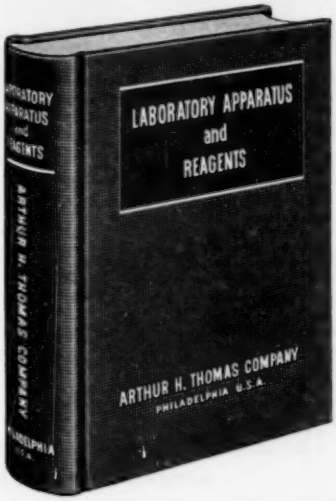

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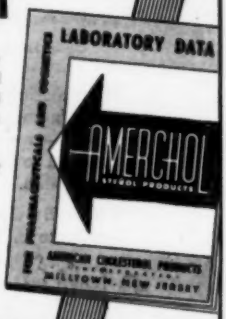

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VOLUME XXXIX | *OCTOBER, 1950* | NUMBER 10 | CONSECUTIVE NO. 20

The Decomposition of Equilin in Oil Solution*,†

By DANIEL BANES, JONAS CAROL, and EDWARD O. HAENNI

Equilin decomposes in oil solutions containing peroxides, and is partially converted to equilenin. The decomposition is accelerated by light.

In an earlier report from this laboratory (1) describing a colorimetric method for the analysis of estrogenic ketosteroid mixtures, it was noted that recoveries of equilin were somewhat low when oil solutions of known concentration were assayed. It was suggested there that the losses could be ascribed to the susceptibility of the ketosteroid to chemical change. This paper presents data elucidating the nature of the decomposition of equilin in oil solution.

EXPERIMENTAL

The sesame oil solutions of estrogenic ketosteroids, whose analyses have been reported earlier (1) and are tabulated in Table I ("Recovered after 1 week"), were again extracted after several months and assayed according to the procedures previously described (1, 2). Comparison of these analytical data (Table I, "Recovered after three months") with those first obtained showed a pronounced loss of equilin.

The reliability of the proposed colorimetric method was tested by applying it to several portions of a freshly prepared solution of estrogens in sesame oil. This solution, containing 0.20 mg. of equilin, 0.70 mg. of estrone, and 0.10 mg. of equilenin per ml. of solution, yielded quantitative recoveries for all three ketosteroids. Losses of equilin in aged oil solutions therefore must have been caused by the continuous action of a degradative agent in the solvent.

To study the course of the decomposition of equilin in greater detail, various solutions of the pure estrogen (m. p. 237–239°, $[\alpha]_D^{25}$ = + 325, 0.5% in alcohol) were prepared, employing U. S. Pharmacopeia grades of sesame oil, corn oil, and peanut oil as solvents. Equilin samples were weighed accurately, dissolved in alcohol, and heated on the steam bath with the respective oils to expel the alcohol. The cooled solutions were diluted with the appropriate oils to yield final concentrations of 0.50 mg. of equilin per ml. of solution. In addition, a fourth solution was prepared to contain 1.0 mg. of equilin and 0.02 ml. of benzyl alcohol per ml. of sesame oil. Each of the mixtures was divided into two portions, one of which was stored in the dark, and the other on the laboratory desk, but not in direct sunlight.

The prepared samples were assayed for equilin periodically. Ketosteroids in 10-ml. portions of solution were extracted by the procedure of Carol and Rotondaro (2), omitting the use of Girard's reagent T to avoid the possible alteration of decomposition by-products during the heating period in glacial acetic acid. Equilin was determined by treating the mixed benzenesulfonates of the extracts

* Received June 12, 1950, from the Chemical Branch of the Division of Medicine, Food and Drug Administration, Federal Security Agency, Washington, D. C.

† Parts of this paper have been presented by Daniel Banes in a dissertation offered in partial fulfillment of the requirements for the degree of Doctor of Philosophy in the Department of Chemistry at Georgetown University.

TABLE I.—ANALYSES OF SESAME OIL SOLUTIONS OF KETOSTEROIDS

Sample No.	Estrone			Equilin			Equilenin		
	Put in Mg./Ml.	Recovered		Put in Mg./Ml.	Recovered		Put in Mg./Ml.	Recovered	
		After 1 Wk.	After 3 Mo.		After 1 Wk.	After 3 Mo.		After 1 Wk.	After 3 Mo.
1	9.7	9.5	9.4	0.0	0.0	0.0	0.0	0.0	0.0
2	4.4	4.1	4.1	2.8	2.4	1.6	1.5	1.6	2.0
3	9.3	8.9	8.9	1.0	0.9	0.6	0.6	0.7	0.7
4	4.7	4.8	4.6	2.3	2.0	1.1	3.5	3.5	3.7

with BQC reagent (1). The course of the decomposition of equilin in oil solutions exposed to light is shown graphically in Fig. 1, which presents the data upon which the idealized curves are based. The nature of the process appeared to be similar for all of the oils used, but the speed of decomposition was accelerated by benzyl alcohol.

After four weeks' storage in the dark, the duplicate oil solutions were assayed. Recoveries of equilin were as follows: sesame oil solution, 95%; peanut oil solution, 88%; corn oil solution, 96%; benzyl alcohol-sesame oil solution, 68%. Thereafter equilin content fell progressively in all samples, perhaps due to repeated exposure to light during sampling.

All of the oil solvents were found to contain peroxides (potassium iodide-starch test), and this fact provided a clue to the nature of the degradative process. Equilin is readily oxidized at the 7,8-ethylenic bond (3). Even when the steroid is subjected to conditions under which unsaturated compounds normally would be hydrogenated, equilin is dehydrogenated to equilenin (4).

When examined spectrophotometrically in ultraviolet light, and when treated with BQC reagent, the oil solution extracts showed evidence of equilenin. Several of these extracts were combined, dissolved in a small volume of benzene, and chromatographed on a 21-Gm. Celite 20-ml. 0.4 *N* sodium hydroxide partition column, following the procedure of Haenni (5). By this means, there was isolated in the third 100-ml. portion of benzene eluate a crystalline substance which exhibited the distinctive ultraviolet and infrared absorption properties of *d*-equilenin (6, 7). Recovery of this substance in the dehydrogenation of equilin at room temperature provides evidence confirming the established view (12) that equilin and equilenin have the same steric configuration in the C/D ring fusion.

The quantities of equilenin formed in the progressive decomposition of equilin in peanut oil, determined by the use of BQC tests before and after esterification (1), are shown in Fig. 2. In all oils, the production of equilenin was proportional to the loss of equilin, but did not account for all of the loss. No effort was made to identify other decomposition products of equilin in oil.

Results obtained in several analyses were checked by independent assay procedures. The infrared spectrophotometric method (6), and a direct base-

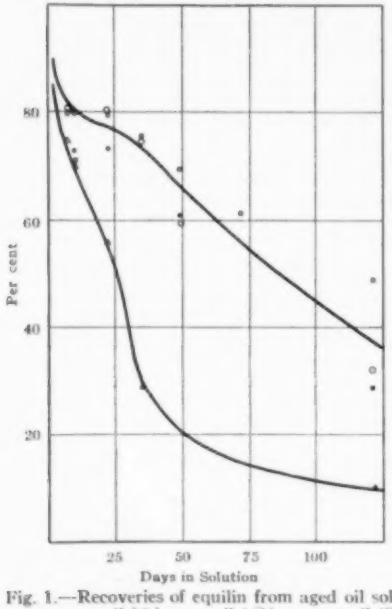

Fig. 1.—Recoveries of equilin from aged oil solutions: sesame oil (●), corn oil (○), peanut oil (□) and sesame oil with benzyl alcohol (△).

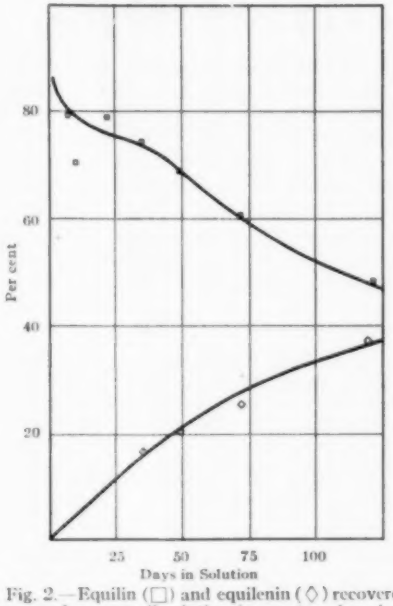

Fig. 2.—Equilin (□) and equilenin (◇) recovered from aged peanut oil solution (percentage based on quantity of equilin put in).

TABLE II.—THE KETOSTEROID CONTENT OF DECOMPOSED EQUILIN SOLUTIONS

Sample	Equilin, Mg./Ml. Put in	Recovered BQC	Recovered I.R.	Lost	Equilenin Found, Mg./Ml. BQC	I.R.	U.V
Sesame Oil	0.50	0.14	0.14	0.36	0.23	0.21	0.23
Peanut Oil	0.50	0.24	0.23	0.26	0.19	0.19	0.19
Corn Oil	0.50	0.17	0.18	0.32	0.18	0.18	0.18
Benzyl Alcohol-Sesame Oil	1.00	0.11	*	0.89	0.48	*	0.47

* Determination vitiated by background interference.

line ultraviolet absorption technique for equilenin were employed. In the latter, the optical densities of suitably diluted sample solutions at 340 mμ, corrected for background interference (half the sum of the densities at 310 and 370 mμ), were compared with the corresponding data for pure standard equilenin solutions. Results, shown in Table II, were in good agreement for all cases in which the respective methods were applicable.

The instability of equilin in oil solution may help to explain the discordant values reported (8–11) for the biological activity of this hormone, from 0.3 to 1.6 times the activity of estrone. The partial conversion of equilin to equilenin represents a progressive loss of estrogenic potency during aging of its oil solutions. Mixed estrogens deriving a substantial proportion of their biological activity from their equilin content are subject to similar losses.

SUMMARY

1. The instability of equilin in oil solutions containing peroxides has been demonstrated.

2. The rate of decomposition of equilin has been shown to be accelerated by light, and also to be accelerated by the presence of benzyl alcohol.

3. Equilenin has been obtained as a degradation product of equilin in oil solution.

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WHO MAKES IT?

The National Registry of Rare Chemicals, Armour Research Foundation, 33rd, Federal and Dearborn Streets, Chicago, Ill., seeks information on sources of supply for the following chemicals:

Perilla oil
Linamarin
Myricin
Biliverdin
Renin
Hypertensinogen
Angiotonin
Pyrithiamine
2,3-Dimethylbutanol-3
Ethyl tannate
N-Nitrosodiethyl amine
Benzopyrene
5,5′-Diethoxythioindigo
Laccase
ρ-Sorbic acid
Phellandral
Thropanic acid
Psothanol
Jaluronic acid
Carvacrol

Iron-Kober Reagent: A More Sensitive Stable Reagent for the Determination of *Alpha*- and *Beta*-Estradiols*

By EDWARD O. HAENNI†

Minute quantities of iron markedly affect the development of characteristic colors of estrogens in the Kober reaction. Detailed study of these effects on the yellow and red phases of the Kober colors produced by α- and β-estradiols resulted in development of a new iron-containing phenolsulfonic acid type reagent for these estrogens. The reagent is characterized by much higher sensitivity for α-estradiol and a lower sulfuric acid content than is usual in Kober reagents. It is stable for months. The diluted reagent is highly sensitive for β-estradiol but does not give a color with α-estradiol. Analytical procedures are presented for the determination of α-estradiol and β-estradiol in mixtures containing up to 30 μg. of total estradiols. Results of analysis of such mixtures including laboratory-prepared oil solutions, show very good precision and accuracy. Analyses of typical commercial α-estradiol preparations (oil solution, aqueous suspension, tablets) yield precise results in good agreement with those obtained by the infrared method. The colorimetric method is presented in conjunction with a simple extraction procedure intended primarily for application to those pharmaceutical α-estradiol preparations essentially free of phenolic estrogens other than the estradiols. Its application in combination with partition chromatography to more complex estrogenic pharmaceuticals will be described in a later report.

A PROCEDURE combining Stimmel's chromatographic technique (1) with the Carol and Molitor method (2) (modified Kober reaction) developed in this laboratory for the determination of α- and β-estradiols failed to yield consistent results due to variation in lots of activated alumina. Although intensive study of the purification and activation of the alumina did not result in production of a satisfactory adsorbent, it did lead to the discovery of the marked effects of minute quantities of iron on the development of characteristic colors in the Kober reaction for estrogens.[1] The present report concerns use of these effects in the development of a more sensitive stable reagent for the determination of α- and β-estradiols.

Although numerous papers (2–12) have been published dealing with the application of Kober type reactions to the determination of estrogens, the significance of the presence of microgram quantities of the ubiquitous iron in such reactions has not been recognized heretofore. This unrecognized variable probably accounts for an occasional high color value for α-estradiol observed in the Carol-Molitor procedure and for erratic results experienced with other Kober procedures for estrogens. The chief difficulties with the Kober reaction, however, are inherent in its bicolor character. The initial yellow phase is better converted to the more specific red phase for most quantitative applications. The development of the yellow phase, its rates of conversion to colorless or red phases and the intensity and stability of the red phase are affected by a number of factors; namely, the identity and concentration of the estrogen, the composition of the reagent, the time and the temperature, as well as the presence of iron. In general the high concentrations of sulfuric acid necessary for good development of the initial yellow color inhibit the conversion to the red phase in varying degrees with the different estrogens. Accordingly, most methods have included a second stage involving dilution of the initial reaction mixture and reheating to develop the red color. However, at the lower sulfuric acid concentrations necessary in the second stage, both the yellow and red color components are destroyed at more or less rapid rates, depending on the estrogen concerned. This fact imposes strict control of time, temperature, volumes of reagent and diluent, and manner of operation as a requirement for satisfactory results.

Another major limitation to the practical application of the Kober reaction is the fact that the necessary heating of estrogenic extracts with the usual reagents produces interfering yellow-brown colors due to the charring effect of the sulfuric acid on nonestrogenic impurities. Such impurities in extracts from pharmaceutical

* Received June 15, 1950, from the Chemical Branch of the Division of Medicine, Food and Drug Administration, Federal Security Agency, Washington, D. C.

† The author wishes to thank his co-workers, Jonas Carol and Daniel Banes, for their collaborative analyses and constant cooperation during this investigation.

[1] Other metals may have similar effects on the Kober reaction. However, because iron is the most likely contaminant the present study has been limited to its effects. These effects can also be demonstrated to occur in the case of phenol-free Kober reagents. In a later paper it will be shown that traces of iron may affect the character as well as the intensity of the color produced with some estrogens.

estrogen preparations may come not only from the estrogenic ingredient but also from solvent residues and the stabilizers, excipients or vehicles used. This interference is a serious problem in the analysis of estradiol preparations, in contrast to ketosteroid preparations, because, first, the concentration of estrogen, as well as the ratio of estrogen to impurities, is commonly of the order of one-tenth as great in the former; and, secondly, there is no reagent for the diols comparable to the Girard reagent which permits quantitative isolation of ketosteroids in sufficient purity for direct colorometric determination (13). Experience in this laboratory confirms the findings of Venning, *et al.* (7), and subsequent investigators (1, 14) that correction for such nonestrogenic impurities based on the ratio of the optical density at the absorption maximum of the red color to the optical density at 420 mμ is satisfactory if the ratio is not too low. Accordingly, it is desirable that the reagent used have a high sensitivity for α-estradiol with a minimum concentration of sulfuric acid.

Two major effects of iron on the Kober reaction with α- and β-estradiols were soon observed. One is the increase in the rate of development and intensity of the initial yellow phase. The second is the increase in the rate of conversion to the red phase. These effects produced by 5 μg. of ferric iron per milliliter in the often-used Cohen-Marrian type of Kober reagent (4) are shown in Fig. 1. Less than 1 μg. of iron per ml. affects the reactions. As the consistent exclusion of such minute quantities of iron from Kober reactions seemed impracticable, it was decided to exploit these effects in developing more sensitive reagents for α- and β-estradiols.

An intensive investigation of all the variables mentioned above was made, including a study of the manner of preparation of many phenolsulfonic acid reagents and their ultimate composition. As a result, reagent A was developed, which permits complete conversion of the yellow phases of both α- and β-estradiols to stable strong red colors in a one-stage heating operation. Absorption spectra of the colors produced are shown in (Fig. 2, *a*). The $E_{525\ m\mu} - E_{420\ m\mu/2}$ values are directly proportional within practical limits to the concentration of estradiol for both the α- and β-isomers, singly and in combination (Fig. 2, *b*). Reagent B (made by dilution of reagent A) develops no color with α-estradiol under conditions of the procedure but produces a strong red color with β-estradiol in a one-stage heating operation (Fig. 3, *a*). The $E_{526\ m\mu} - E_{420\ m\mu/2}$ values are directly proportional to the

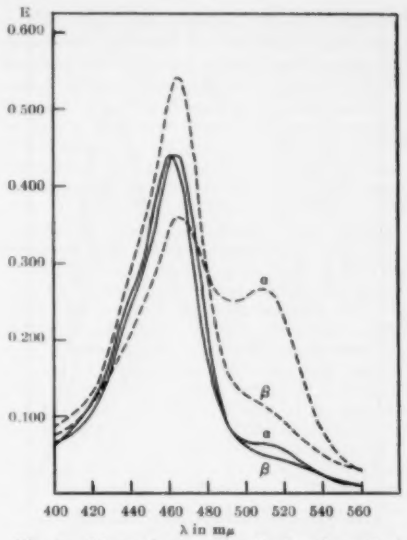

Fig. 1.—Absorption spectra of colors formed with 20 μg. samples of α- and β-estradiols on heating ten minutes with 5 ml. of Cohen-Marrian reagent (solid line) and with the same reagent containing 5 μg. of ferric iron per ml. (broken line).

β-estradiol concentration even in the presence of more than twice the quantity of α-estradiol that would be found under the conditions of procedure B (Fig. 3, *b*). Accordingly, α-estradiol is determined as the difference between the total estradiols from procedure A and the β-estradiol from procedure B.

EXPERIMENTAL

The empirical character of the Kober reaction and the many variables affecting it necessitated accumulation of a mass of detailed data in the development of the present method. Space limitations do not permit its inclusion in this report. Accordingly, only a few of the basic experiments related to the characteristics of reagents A and B are reported, together with some of the general findings regarding the Kober reaction with α- and β-estradiols.

Composition of the Reagents.—Reagent A contains 30 μg. of ferric iron per ml. in a phenolsulfonic acid solution with excess phenol. The solution is made with only 42% by volume (55% by weight) of concentrated sulfuric acid, the minimum content which yields a stable fluid reagent with satisfactory chromogenic properties.[1] Ferrous iron is not as efficient an activator as ferric iron, possibly acting only after oxidation *in situ*. The quantity of ferric iron is not particularly critical. The concentration adopted is such as to develop the complete reaction without further effect due to fortuitous con-

[1] Higher ratios of phenol to sulfuric acid have the desirable effects not only of shifting the absorption maxima of the red colors with α- and β-estradiols to higher wave lengths but also of bringing them closer together. However such reagents soon crystallize.

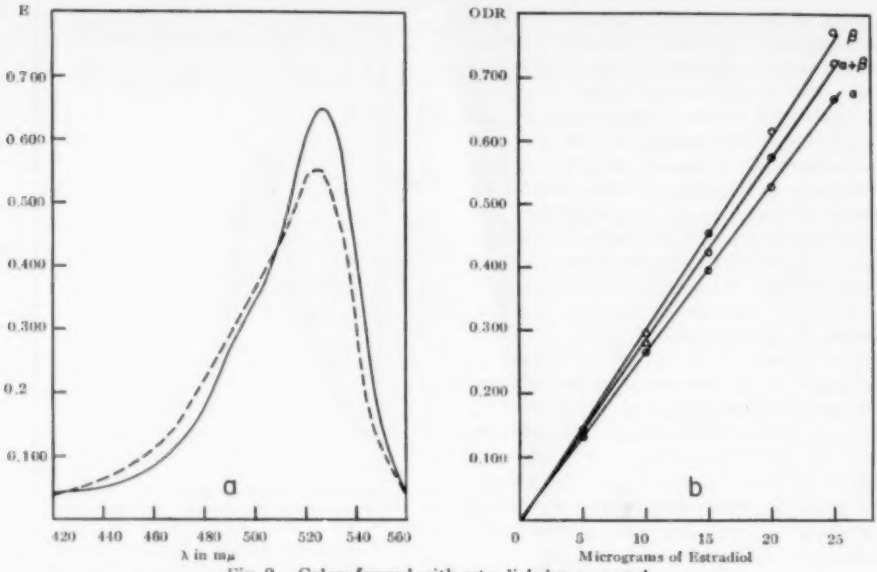

Fig. 2.—Colors formed with estradiols by reagent A.
a, Absorption spectra for 20 μg. of α-estradiol (broken line) and β-estradiol (solid line) in procedure A.
b, Optical density relation (ODR) $E_{525\ m\mu} - \frac{E_{420\ m\mu}}{2}$ versus estradiol concentration (μg. per 5 ml.) in procedure A for α-estradiol, β-estradiol, and mixtures containing 50% of each.

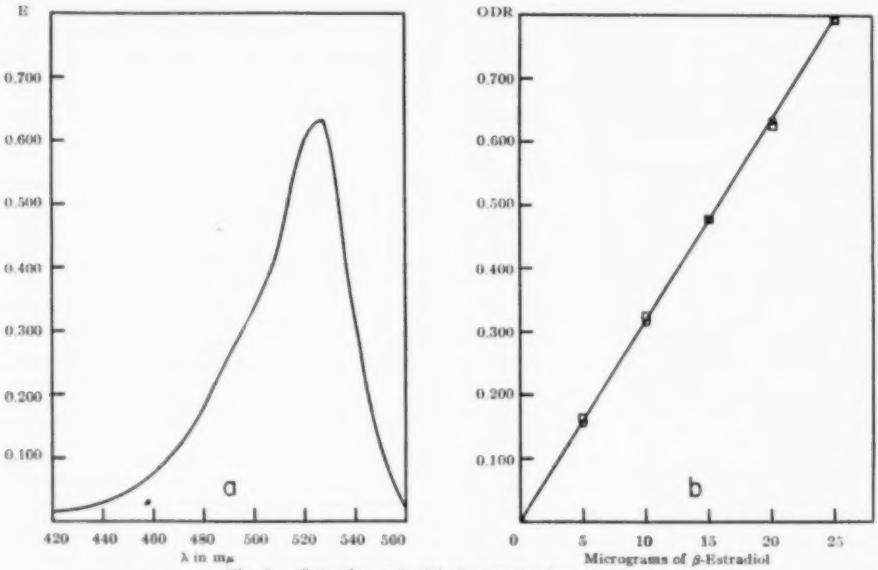

Fig. 3.—Color formed with β-estradiol by reagent B.
a, Absorption spectra for 20 μg. of β-estradiol in procedure B.
b, Optical density relation (ODR) $E_{525\ m\mu} - \frac{E_{420\ m\mu}}{2}$ versus β-estradiol concentration (μg. per 5 ml.) in procedure B for β-estradiol (○) and β-estradiol plus 50 μg. of α-estradiol (□).

tamination with traces of additional iron. Reagent B is prepared by dilution of reagent A with sufficient water to inhibit the development of color with α-estradiol and still retain a high sensitivity for β-estradiol.

Reproducibility, Stability, and Sensitivity of Reagents.—Twelve batches of reagent A, prepared at intervals during eighteen months from various lots of phenol and sulfuric acid, have shown the following limits of the $E_{525\ m\mu} - E_{420\ m\mu/2}$ values for 20 μg. of α- and β-estradiols, respectively: 0.507 to 0.537, and 0.585 to 0.649. Batches of reagent B prepared from these gave limiting values of 0.594 to 0.632 for $E_{526\ m\mu} - E_{420\ m\mu/2}$ with 20 μg. of β-estradiol. The stability of reagent A is indicated by the fact that three batches prepared at different times and used intermittently during the storage period showed the following changes in the values with α-estradiol, respectively: from 0.514 to 0.515 after seventeen months, from 0.513 to 0.496 after seventeen months, and from 0.513 to 0.509 after thirteen months. Moreover, in contrast to the usual phenol-sulfonic acid reagents, reagent A shows little decrease in fluidity on storage. These properties permit preparation of large batches of the reagent and, in conjunction with the small quantity used in a determination, make it possible to quickly prepare in one batch a supply for a long period. The ease of preparation of reagent B makes its stability a matter of less importance. However, it yields satisfactory results for at least several weeks after preparation.

The comparative sensitivity for α-estradiol of the procedures proposed for its determination in the presence of β-estradiol is indicated by the $E_{1\ cm.}^{1\%}$ values of the yellow color [Umberger-Curtis (12)] and red color [Carol-Molitor (2) and present procedure] at the wave lengths used in the methods. These values are, respectively, 1050, 550, and 1350. The corresponding values for the red colors obtained in the procedures for the determination of β-estradiol are, respectively, 640, 930, and 1560.

Color Development-Heating Time Relation of Reagents with α- and β-Estradiols.—The rate of change of color intensity with time of heating at the time selected is of great significance in determining the precision of Kober methods, particularly for mixed estrogens. In the development of the present procedures some sensitivity was sacrificed in the interest of producing a red color with both estradiols showing little change within time limits that can be easily controlled. Figure 4 (*a*) shows the rate of color development of both estradiols in procedure A, while Fig. 4 (*b*) shows the relation for β-estradiol in procedure B. The period of reaction at room temperature in procedure A is necessary to produce maximal consistent color development with β-estradiol. Its control is not critical for β-estradiol and it may be omitted in the absence of β-estradiol without affecting the color developed by α-estradiol. The times stated for cooling and diluting are not at all critical but are given as a matter of convenience of operation.

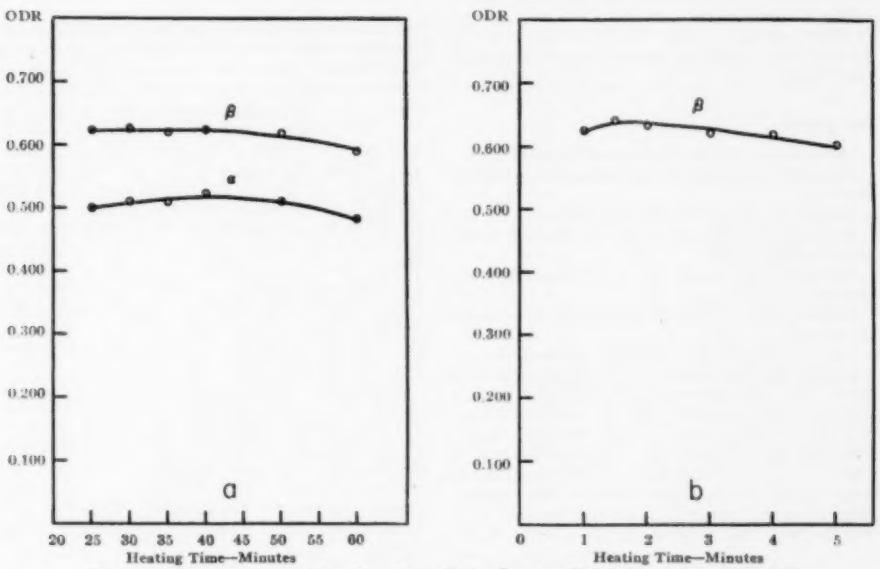

Fig. 4.—Color development–heating time relation for estradiols with reagents A and B.

a, Optical density relation (ODR) $E_{525\ m\mu} - \frac{E_{420\ m\mu}}{2}$ versus heating time in procedure A for α-estradiol and β-estradiol (20 μg. in each experiment).

b, Optical density relation (ODR) $E_{526\ m\mu} - \frac{E_{420\ m\mu}}{2}$ versus heating time in procedure B for β-estradiol (20 μg. in each experiment).

Stability of Colors.—The colors are stable for several hours after dilution with the concentration of sulfuric acid prescribed. The red color from β-estradiol is more stable than that from α-estradiol. Figure 5 shows the effect of the concentration of sulfuric acid on the stability of the α-estradiol color. In this experiment the full color of α-estradiol was developed in a large portion of reagent, and at room temperature the change in the optical density with time of aliquots diluted to the same volume with various concentrations of sulfuric acid was measured. The diluted solution in procedure A contains about 39% by volume of sulfuric acid.

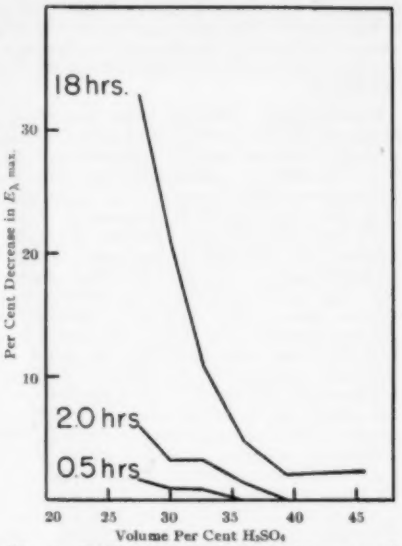

Fig. 5.—Fading of color formed with α-estradiol versus concentration of H_2SO_4 on standing at room temperature. Concentration of H_2SO_4 expressed as per cent by volume of H_2SO_4 A. C. S. in the total volume of the diluted solution. Initial $E^{1\ cm.}_{\lambda\ max.} = 0.42$.

METHOD

Apparatus

A spectrophotometer or photometer capable of indicating spectral bands of the order of 2 mμ or less in the region of 400–700 mμ. (A Beckman Quartz Spectrophotometer fitted with matched 1-cm. absorption cells was used in this investigation.)[3]

Separatory funnels, 125 ml., with well-fitting stopcocks lubricated only with water.

Silicon carbide, 20 mesh (obtainable from the Carborundum Company, Niagara Falls, N. Y.).

Clean, dry, light rubber finger stalls, small or medium size (obtainable from drug supply houses or most drug stores).

Clean, dry, solid glass beads—4 to 5 mm. diameter.

Three burettes with stopcocks lubricated only with reagent. The orifice of one burette should be enlarged, if necessary, to deliver 1 ml. of reagent A in thirty seconds or less. Protect reagents in the burettes from absorption of moisture with suitable guard tubes.

Reagents

Skellysolve C or high boiling petroleum ether.

Dilute sodium hydroxide solution—2 *N* (approximate).

Sodium carbonate solution—10% w/v.

Sulfuric acid solution—mix 35 volumes H_2SO_4 with 65 volumes H_2O.

Sodium sulfate —A. C. S. grade anhydrous powder.

Benzene—A. C. S. grade, redistilled in all-glass apparatus.

Carbon tetrachloride—A. C. S. reagent grade.

Reagent A (Stock Iron-Kober Reagent)—Dissolve 1.054 Gm. of $FeSO_4 \cdot (NH_4)_2SO_4 \cdot 6H_2O$ (Mohr's salt) in about 20 ml. H_2O, add 1 ml. H_2SO_4 and 1 ml. 30% H_2O_2. Mix, heat until effervescence ceases, and dilute to exactly 50 ml. To 3 volumes of iron solution in a volumetric flask add H_2SO_4 (A. C. S. reagent grade) with cooling to make 100 volumes. Redistill U. S. P. phenol[4] discarding the first 10% and the last 5%. Collect the distillate with exclusion of moisture in a dry tared flask of about twice the volume of the phenol and equipped with a glass stopper. Place the stoppered flask in an ice bath to solidify the phenol, breaking the top crust with a glass rod to be sure of complete crystallization. Dry and weigh the flask. Add to the phenol 1.13 times its weight of the iron-sulfuric acid solution, stopper the flask and allow to stand without cooling but with occasional mixing until the phenol is liquefied (approximately thirty minutes or less). Shake the mixture vigorously until homogeneous and allow to stand in the dark sixteen to twenty-four hours. Add to the mixture 23.5% of its weight of a solution of 100 volumes of H_2SO_4 in 110 volumes of H_2O. Shake the mixture vigorously to homogeneity. Transfer to dry glass-stoppered bottles. Stored in the dark and protected from absorption of moisture, this reagent is stable for months.

Reagent B—To a carefully measured volume of reagent A in a glass-stoppered graduated cylinder add 0.45 volume of H_2O, mix, cool, and transfer to dry glass-stoppered bottles. Store in the dark and protect from absorption of moisture. Inspect before use and disperse any flocculent precipitate by vigorous swirling of the reagent. With this precaution the reagent may be used satisfactorily for weeks.

Standard Solution

α-Estradiol Standard.—Twenty micrograms/ml. Dissolve exactly 10 mg. α-estradiol in alcohol and dilute with alcohol to 100 ml. in a volumetric flask. Pipette exactly 10 ml. of this solution into a 50-ml. volumetric flask and make to volume with alcohol. Store in the dark.

[3] If the instrument available requires larger volumes of solution, the procedures may be extended in range to at least 50 μg. of estradiols without any other change than doubling or tripling the quantities of reagent and diluent sulfuric acid in both procedures. Alternatively the specified 1 ml. of reagent may be diluted with greater proportions of sulfuric acid, provided the consequent slight shift of the absorption maxima of the red colors toward shorter wave lengths is taken into account.

[4] Use of 1 lb. of phenol and 1 L. flasks has been found convenient.

β-Estradiol Standard.—Twenty micrograms/ml. Prepare in the same way from β-estradiol. Store in the dark.

Sample Solution

Transfer a portion of the oil (or the estradiol extract from tablets or aqueous suspensions, dissolved in 5 ml. of ether or chloroform) containing ca. 75–250 μg. of total estradiols to a 125-ml. separatory funnel with 25 ml. Skellysolve C. Add 10 ml. dilute sodium hydroxide, shake vigorously for two minutes and allow the two layers to separate completely. Transfer the aqueous layer to a second 125-ml. separatory funnel containing 5 ml. of carbon tetrachloride, avoiding transfer of any insoluble matter at the interface. Repeat the extraction with two additional 10-ml. portions of dilute sodium hydroxide, adding each to the second funnel. (The sodium hydroxide extraction should be completed as quickly as possible. Long standing in strongly alkaline solution might cause some decomposition of estradiols.) Discard the Skellysolve solution. Shake the alkaline solution vigorously with the carbon tetrachloride and allow to separate. Draw off the carbon tetrachloride layer closely into a third separatory funnel and wash with 5 ml. of dilute sodium hydroxide. Discard the carbon tetrachloride and add the alkali to the solution in the second funnel, rinsing the third funnel with a little water. Acidify the solution with the sulfuric acid solution (ca. 8 ml.), cool and shake vigorously with 20 ml. benzene for one minute. (In this and subsequent shake-outs ignore any slight turbidity persisting after five minutes standing if the interface is sharply defined.) Transfer the lower layer to the third separatory funnel and repeat the extraction with another 20 ml. benzene. Discard the acid layer. Beginning with the first benzene extract wash the extracts in succession with two 5-ml. portions of dilute sodium carbonate solution and two 5-ml. portions of water. Discard the aqueous layers, drawing off the last one as closely as possible. Drain the first benzene extract into a dry 100-ml. beaker. Sprinkle about a gram of anhydrous sodium sulfate into the benzene and swirl the benzene until it is *entirely clear*. Decant into a 50-ml. volumetric flask avoiding transfer of any of the sodium sulfate mixture. (Do not allow the stirring rod used in decanting to come in contact with the sodium sulfate.) Rinse the second funnel with the other benzene extract from the third funnel and add this extract to the beaker. Repeat the clarification by swirling, adding further small portions of sodium sulfate, if necessary. Transfer the benzene to the volumetric flask. Rinse the funnels in succession with two 4-ml. portions of benzene, clarifying each portion in the beaker and decanting into the flask. Make to volume with benzene and mix.

Procedure A.—Transfer in duplicate to dry 18 x 150 mm. test tubes: (*a*) an aliquot of the sample solution containing 15–25 μg. of total estradiols;[5] (*b*) 1 ml. of α-estradiol standard; and (*c*) 1 ml. of β-estradiol standard. Add several pieces of silicon carbide to each tube and rapidly evaporate the solvent in the steam bath (do not use a current of air) until ebullition from the boiling stones just stops. Instantly remove the tube, wipe dry quickly and transfer to an efficient vacuum desiccator. Keep the tube in the desiccator connected to the vacuum at least one hour.[6] To each tube and to a blank tube add a glass bead and measure into each tube from a burette 1 ml. of reagent A, quickly wiping the outside of the tip with a piece of absorbent paper before each addition. Immediately cap the tubes with rubber finger stalls and allow to stand for thirty minutes, shaking the tubes vigorously at five-minute intervals. Place in a boiling water bath for thirty-five minutes, removing and shaking each tube for a few seconds after the first five minutes. Transfer to an ice bath for two minutes, then remove and add from a burette exactly 4 ml. of the sulfuric acid solution. Allow to stand five minutes and mix by shaking, first gently, then vigorously, to homogeneity. Measure the optical densities of the sample and standards relative to the blank at 525 mμ (midpoint between the maxima for α-estradiol and β-estradiol) and at 420 mμ making any necessary corrections due to cell variation.

$$\text{Micrograms of total estradiols (calculated as } \alpha\text{-estradiol) in aliquot} = 20 \times \frac{E_{525\ m\mu}\ \text{sample} - (E_{420\ m\mu}\ \text{sample}/2)}{E_{525\ m\mu}\ \alpha\text{-estradiol standard} - (E_{420\ m\mu}\ \alpha\text{-estradiol standard}/2)} = T_a$$

$$\text{Micrograms of } \beta\text{-estradiol (calculated as } \alpha\text{-estradiol) in aliquot} = \text{Micrograms } \beta\text{-estradiol in aliquot (from procedure B)} \times \frac{E_{525\ m\mu}\ \beta\text{-estradiol standard} - (E_{420\ m\mu}\ \beta\text{-estradiol standard}/2)}{E_{525\ m\mu}\ \alpha\text{-estradiol standard} - (E_{420\ m\mu}\ \alpha\text{-estradiol standard}/2)} = B_a$$

$$\text{Micrograms of } \alpha\text{-estradiol in aliquot} = T_a - B_a$$

Procedure B.—Transfer in duplicate to dry 18- x 150-mm. test tubes: (*a*) an aliquot of the sample solution containing 15–25 μg. of total estradiols;[5] and (*b*) 1 ml. of β-estradiol standard. Add several pieces of silicon carbide to each tube, evaporate the solvent and dry the residue exactly as described in procedure A. To each tube and to a blank tube add from a burette 1 ml. of reagent B, quickly wiping the outside of the tip with a piece of absorbent paper before each addition. Immediately cap the tubes with rubber finger stalls and place in a boiling water bath for exactly two minutes, shaking the tube after thirty seconds for a few seconds without removing from the bath. Transfer to an ice bath for two minutes, then remove and add from a burette exactly 4 ml. of the sulfuric acid solution. Mix by shaking vigorously to homogeneity. Measure the optical densities of the sample and standard relative to the blank at 526 mμ, (maximum for β-estradiol) and at 420 mμ, making any necessary corrections due to cell variation.

[5] It is evident from the data reported that the quantity limits are not restrictive. However, the quantities suggested are convenient in taking aliquots and yield color intensities in a range of high accuracy for the more critical α-estradiol determination.

[6] The dry estrogens are sensitive to excessive heating in the presence of air. The technique described is satisfactory for handling the smallest quantities without loss.

$$\text{Micrograms of } \beta\text{-estradiol in aliquot} = 20 \times \frac{E_{526\ m\mu}\ \text{sample} - (E_{420\ m\mu}\ \text{sample}/2)}{E_{526\ m\mu}\ \beta\text{-estradiol standard} - (E_{420\ m\mu}\ \beta\text{-estradiol standard}/2)}$$

DISCUSSION

The precision and accuracy of the method are indicated by the results shown in the tables. Table I shows the results obtained in the analysis of mixtures of α- and β-estradiols in alcohol solutions. Table II shows results obtained with synthetic solutions of estradiols in sesame oil by three analysts in this laboratory, two having had no prior experience with this particular procedure. Table III includes typical results obtained in analysis of commercial preparations by this procedure compared with the results obtained by the infrared method (15).

The assay data show that the procedures yield precise and accurate results for α- and β-estradiols in mixtures and in pharmaceutical preparations free of other estrogenic steroids.

The extraction and washing procedure, developed from that of Carol and Rotondaro (16), separates the weakly phenolic estrogens from substances in pharmaceutical preparations likely to interfere in the colorimetric determinations.

In common with all related reagents for α-estradiol, reagent A develops some color with other estrogenic steroids. Banes (13) applied a modification of reagent A to the determination of estrone, correcting for the blue and orange colors due to equilin and equilenin, respectively, by means of independent colorimetric methods for these two. α-Dihydroequilin yields a red color with reagent A of about one-half the intensity of that given by α-estradiol, while α- and β-dihydroequilenins yield weak orange colors. With reagent B α-dihydroequilin develops an intense yellow color which

Table I.—Analysis of Estradiol Mixtures in Alcohol

Sample	α-Estradiol Micrograms Present	α-Estradiol Micrograms Found	α-Estradiol Av. % Recovery	β-Estradiol Micrograms Present	β-Estradiol Micrograms Found	β-Estradiol Av. % Recovery
1	20.0	19.8	100	1.0	1.1	100
		20.2			0.9	
2	15.0	15.3	101	5.0	5.0	99
		15.1			4.9	
3	10.0	9.9	101	10.0	9.9	98.5
		10.3			9.8	
4	5.0	4.8	99	15.0	14.8	99
		5.1			14.8	
5	1.0	0.7	70	20.0	20.0	100
		0.7			20.0	
6	5.0	4.8	96	5.0	5.1	102
		4.8			5.1	
7	25.0	26.2	105	5.0	5.6	112
		26.5			5.6	
8	10.0	9.5	96.5	20.0	20.0	100
		9.8			20.0	

Table II.—Analysis of Estradiol Mixtures in Oil

Analyst	Sample I[a] α-Estradiol Found, μg./Ml.	Sample I[a] α-Estradiol Average Recovery, %	Sample I[a] β-Estradiol Found, μg./Ml.	Sample I[a] β-Estradiol Average Recovery, %	Sample II[b] α-Estradiol Found, μg./Ml.	Sample II[b] α-Estradiol Average Recovery, %	Sample II[b] β-Estradiol Found, μg./Ml.	Sample II[b] β-Estradiol Average Recovery, %
1	20.0	100	5.1	102	17.7	100	4.0	100
	20.1		5.1		18.2		4.0	
2	20.4	102	5.1	102	17.9	99	3.9	97.5
	20.3		5.1		17.9		3.9	
3	19.9	99.5	5.0	100	18.1	101	4.0	101
	..		5.0		..		4.1	

[a] Sesame oil solution—20.0 μg./ml. α-estradiol. 5.0 μg./ml. β-estradiol.
[b] Sesame oil solution—18.0 μg./ml. α-estradiol. 4.0 μg./ml. β-estradiol.

Table III.—Analysis of Commercial Estradiol Preparations

	Declared α-Estradiol Content	Colorimetric Method, Estradiols Found α	β	Total	Infrared Method, α-Estradiol Found
Oil solution—α-estradiol, chlorobutanol, sesame oil	0.050 mg./cc.	0.046	0.001	0.047	0.042
		0.047	0.001	0.048	
Aqueous suspension—α-estradiol, chlorobutanol, acacia, phenol, saline solution	0.28 mg./cc.	0.269	0.017	0.286	0.262
		0.270	0.017	0.287	
Tablets—α-estradiol with excipients	0.022 mg./tab.	0.014	0.001	0.015	0.014
		0.014	0.001	0.015	

serves as an indicator of its presence but does not significantly interfere in the determination of β-estradiol.[7] β-Dihydroequilenin gives a weak orange color with reagent B, equilin a weak blue-green. Estrone, equilenin and α-dihydroequilenin do not yield colors with reagent B.

The procedure presented for determination of α- and β-estradiols is intended primarily for the analysis of estrogenic pharmaceutical preparations essentially free of other phenolic estrogens. In a subsequent paper the author will describe the application of the reagent in conjunction with a simple partition chromatographic method to the determination of α- and β-estradiols in estrogenic mixtures including the other diols and the ketosteroids.

SUMMARY AND CONCLUSIONS

1. The effects of minute quantities of iron on the development of characteristic Kober colors with estrogens have been studied.

2. A stable, highly sensitive iron-Kober reagent has been developed, producing maximal red colors with α- and β-estradiols in a single-stage heating operation. The diluted reagent is highly sensitive for β-estradiol even in the presence of α-estradiol.

3. Results of analysis of mixtures of α- and β-estradiols ranging up to 30 μg. and including as little as 1 μg. of one isomer in the presence of 20 μg. of the other show very good precision and accuracy.

4. A procedure for the determination of α- and β-estradiols in oil solution is presented. Results of analysis of such solutions by different analysts also show very good reproducibility and accuracy.

5. Application of the method to typical commercial α-estradiol preparations (oil solution, aqueous suspension, tablets) shows that the method gives results concordant with those obtained by the infrared spectrophotometric method.

6. The method is not applicable to preparations containing appreciable proportions of other phenolic estrogens.

[7] Reagent B readily permits visual detection of less than 1 μg. of β-estradiol. One microgram in the presence of as much as 100 μg. of α-dihydroequilin is readily observed by diluting the colored solution with two volumes of alcohol, which destroys the yellow color produced by α-dihydroequilin.

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SECOND PAN-AMERICAN CONGRESS OF PHARMACY

The Second Pan-American Congress of Pharmacy will take place in the City of Lima, Peru, December 1–8, 1951. Announcement of the date has just been made through Dr. Hector Zayas Bazan, the General Secretary of the Congress. Members of the AMERICAN PHARMACEUTICAL ASSOCIATION who attended the First Congress, which was held in Cuba in December, 1948, will recall the friendly relations which were fostered during this meeting, and it is anticipated that a large number of pharmacists from the United States will plan to attend the Second Congress.

As the program for the Congress is developed by the Special Committee which has been appointed for that purpose, further announcements will be made in the JOURNAL.

Toxicity and Repellency to Rats of Actidione*

By ROBERT TRAUB†, JAMES B. DeWITT, JACK F. WELCH, and DORIS NEWMAN‡

The antibiotic actidione was found to be highly repellent to laboratory rats and to significantly reduce gnawing attacks upon treated paperboards. Rats refused to accept food or water containing this material even under conditions of acute starvation and died of starvation and thirst rather than accept water containing 1.0 mg. of actidione per liter. The compound is highly toxic to rats with the minimum lethal dose by oral administration being approximately 1.0 mg./Kg. body weight. Paperboard treated with the compound resisted gnawing attacks by specially trained and motivated rats for periods of two hundred hours, although similar untreated boards were pierced within thirty to sixty minutes.

EARLY in 1947, workers in the Research Laboratories of the Upjohn Company described a new antibiotic produced by certain strains of *Streptomyces griseus* (1). The compound was described a di-ketone, and was said to have the empirical formula of $C_{27}H_{42}N_2O_7$. Subsequent publications (2–4) stated that the compound was a mono-ketone with the formula $C_{15}H_{23}NO_4$. The crude product was stated to be highly irritating to the skin, and the toxicity (LD_{50}) to various animal species was found to be: laboratory mice, 150 mg./Kg. (I. V.); guinea pigs, 60 mg./Kg. (S. C.); rabbits, 17 mg./Kg. (I. V.); cats, 4 mg./Kg. (I. P.); rats, 2.7 mg./Kg. (S. C.); and 2.5 mg./Kg. (I. V.). Others have remarked on the species variability regarding toxicity (5). Recently, actidione has been found to offer promise as a fungicide in the control of plant diseases (6).

During the course of experiments at the Army Medical Department Research and Graduate School on the efficacy of various chemicals in the chemotherapy of parasitic infections, it was noted that mice and rats would not drink water containing actidione. Investigation showed that solutions of this antibiotic were highly repellent to rats even at extreme dilutions, and that food dipped in such solutions was rejected by the animals. Studies at the Patuxent and Denver laboratories of the Fish and Wildlife Service[1] extended these observations and confirmed the previous findings as to the repellent activity of the compound. Data on the acceptability, repellent activity, and toxicity of actidione are presented in this report.

MATERIALS AND METHODS

Toxicity Studies.—Toxicity to laboratory rats was determined by administration of aqueous solutions or suspensions by stomach tube. Concurrent studies on the efficacy of actidione in experimental amebiasis in rabbits provided an indication of the toxic reactions following subcutaneous, intraperitoneal, and intramuscular inoculation of a total dose of from 2.5 mg /Kg. to 15 mg./Kg. of actidione, applied within a period of from five to seven days.

Acceptance Tests.—An index of the repellency to rats of actidione was obtained by offering laboratory animals water or food containing varying amounts of the compound. In initial experiments, individual and pairs of rats were offered standard bottles containing aqueous solutions of the following concentrations (mg./L.): 0.1, 1.0, 5.0, 10.0, 25.0, 50.0, 100.0, and 200.0. No other source of water was available, but untreated food was placed in the cages. In some experiments an alternative of untreated water was offered moribund animals.

Other animals were deprived of food for three days and then offered food which had been dipped in water containing 5 to 200 mg./L. of actidione and then allowed to dry exposed to air for two weeks. After having access to no other food for periods of one hour to three days, these rats were simultaneously given untreated food.

Final determination of the effects of actidione on the acceptance by rats of treated food was made when individually caged animals were given two food cups; one containing 20 Gm. of ground laboratory chow, and the other containing 20 Gm. of food treated to contain 1.0, or 0.5% of actidione. Observations were made over a four-day period. Simultaneously, another group of rats was given a diet containing 0.5% actidione with no other food available.

Laboratory rats were trained and motivated to attack paperboard panels by requiring them to gnaw through such materials in order to obtain food. Test panels consisting of five plies of 16-lb. kraft paper were coated with a suspension of actidione in lacquer to give a final concentration of 0.05 Gm. of actidione per square inch of surface, and exposed to attack by the rats. To insure maximum activity, test animals were replaced at hourly intervals until penetration of the barrier was achieved.

* Received May 27, 1950, from the Army Medical Department Research and Graduate School, Washington D. C., and the Fish and Wildlife Service of the Department of the Interior, Laurel, Md.; Denver, Colo.

† Major, M. S. C.

‡ With the technical assistance of Ervin Bellack and William G. Jahnes.

[1] These studies were made under a contract with the Quartermaster Corps, Department of the Army. Acknowledgment is made of the cooperation of Dr. Ray Treichler, of the Office of the Quartermaster General, and to Capt. Robert Koenig, M.C., of the Department of Parasitology, AMDRGS. Actidione was obtained from the Upjohn Co., Kalamazoo, Mich. Microscopic studies of the pathology of actidione were made by Major T. C. Jones, V.C., under the direction of Brigadier General R. O. Dart, of the Armed Forces Institute of Pathology.

Because it soon became apparent that the compound was irritating to the animals, it was decided to incorporate it in the test panels, rather than apply it as a surface coat. Test panels consisting of 10 plies of paper were prepared in which the second ply was coated with actidione at a concentration of 0.05 Gm./sq. in. Since these panels were quite resistive, the limited number of trained rats available made it impossible to replace the animals each hour. Accordingly, fresh animals were placed in the cages at irregular intervals. Following contact with the test barrier, each animal was held for observation for a period of one week.

A dual-treated barrier technique was also employed in certain tests, utilizing barriers Nos. 6 and 10 (referred to in Table I). These barriers were cut to a size so that when attached to a piece of cardboard having the standard barrier dimensions, they covered half of the barrier surface. Ten sheets of kraft paper, laminated with latex, were mounted on the other half to complete the barrier. The surface of the latter half was coated with sodium silicate in a concentration of 0.1 Gm./sq. in., and the barriers were shaped to simulate the edge of a carton. Exposure to attack was carried out as outlined above.

RESULTS

Toxicity.—The following results were obtained after oral administration of actidione to laboratory rats:

Dosage Level, Mg./Kg.	No. Killed	Time until Death
200.0	2/2	5 hr.
150.0	2/2	5 hr.
100.0	2/2	5 hr.
75.0	2/2	5 hr.
50.0	2/2	ON
25.0	2/2	ON
15.0	2/2	ON
10.0	2/2	ON
7.5	2/2	ON; 26 hr.
5.0	2/2	ON; 24 hr.
2.5	2/2	24 hr.; 25 hr.
2.0	2/2	ON; 23 hr.
1.5	2/2	25 hr.
1.0	1/2	25 hr.
0.75	0/2	Survived
0.50	0/2	Survived

ON—indicates the animals died during the night following dosing.

All animals which received the higher doses had bloody urine and profuse watery feces.

Rabbits apparently are more resistant to actidione than are rats. Nine out of 10 rabbits in each instance survived dosages of 2.5 mg./Kg. and 4.5 mg./Kg. administered by intraperitoneal injection. This mortality rate compares with that in untreated and uninfected control rabbits on a similar diet lacking greens. Only 7 of 10 rabbits survived intramuscular inoculation of 3.5 mg./Kg. actidione. Susceptibility of rabbits to actidione varied considerably. One of two rabbits in each instance survived intraperitoneal injection of 9 mg./Kg. and 15 mg./Kg., respectively. Preliminary studies on the pathology of rabbits inoculated with actidione indicate that the following symptoms may be somewhat characteristic: loss of weight, enteritis, small hemorrhagic areas in the intestines and necrotic areas about the site of intramuscular injection. However, the Armed Forces Institute of Pathology states *in litt.* that there are no distinctive histologic features which can be attributed to the effect of this drug. At least it is apparent that no lesions have been discovered which explain the symptoms described in these animals. Several intercurrent situations are recognized and are of interest, but do not appear to add significantly to the study of the action of this drug.

Acceptability.—Individually caged laboratory rats failed to consume any measurable quantity of an aqueous solution containing 200 mg. actidione per liter, even though no other source of water was available, during an eleven-day test period. Normal (untreated) food was furnished *ad libitum*, but food consumption dropped from 10.3 Gm./animal during the first twenty-four hours to 2.7 Gm./animal during the second twenty-four hours. No food was consumed after forty-eight hours, and all animals died on the eleventh day of the test. Observed symptoms were extreme emaciation (animals lost an average of 38% of body weight during the first seven days) and bloody areas on the nose and front feet.

Similar results were obtained with solutions containing as little as 1.0 mg./L. A solution containing 0.1 mg./L. appeared markedly repellent, but appreciable amounts were taken overnight. Rats which had refused to drink solutions containing 5.0 and 1.0 mg./L. for three or four days avidly drank normal water offered at that time (Fig. 1) and recovered uneventfully.

No weighable amount of treated food was eaten by rats offered laboratory chow containing 0.5% to 1.0% of actidione. Untreated food simultaneously available was eaten only during the first twenty-four hours, very little being accepted after that period. The nose and front feet were bloody

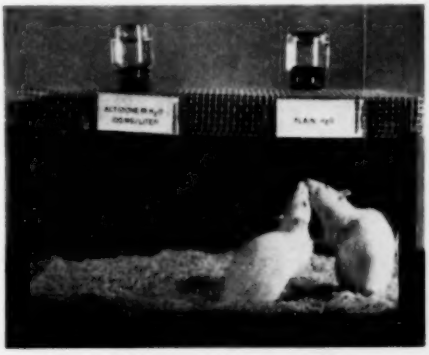

Fig. 1.—Relative acceptance by rats of actidione solution and water. Photograph taken fifteen minutes after animals were given water after a four-day period during which the actidione solution was the only available liquid.

At the left, actidione in water (100 mg./liter). At the right, plain water.

at the end of forty-eight hours and all animals were dead at the end of ten days. Similar results were obtained with rats on a diet containing 0.5% of the antibiotic, when no other food was available. These animals rapidly lost weight, and all were dead by the tenth day.

Rats which had been given normal water, but were limited to food which had been dipped in water containing 50 to 200 mg. of actidione per liter, died within seven to ten days after being placed on the diet. Bloody noses and front feet were again characteristic. Rats which had been starved for three days would approach such treated food when offered, but not so quickly as for untreated food. The treated food was generally quickly expelled from the mouth. Following this, the animals would rub their noses with their feet, or against the cage. After a few more attempts at eating, with similar results, the animals would ignore the food. Some became vile-tempered and fought constantly. As a rule, when untreated food was offered subsequent to rejection of the actidione-treated pellets, such food was eaten avidly.

Rats were observed to feed rather regularly on pellets which had been dipped in water containing 10 to 40 mg. of actidione per liter. This was done with less scurrying about and rubbing of noses than in the previous test, but even so, most of the food was apparently rejected. Much of it was wasted and scattered about during the attempts to eat the pellets. It was observed also that the animals drank large amounts of water while attempting to feed on the treated baits. Four out of five rats failed to survive three days of starvation followed by seven days on a diet that had been dipped in water containing 10.0 mg. of actidione per liter.

Barrier Studies.—Specially trained rats failed to damage barriers which had been coated with actidione. These tests were discontinued at the end of fifteen hours' exposure, since it appeared that the irritant properties of the compound were affecting the reaction of the animals. Subsequent tests, utilizing barriers in which the actidione had been added to an interior layer, are summarized in Table I.

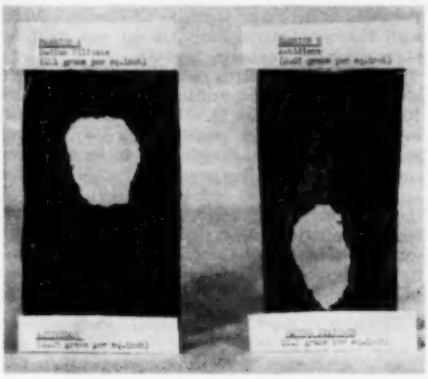

Fig. 2.—Comparison of resistance to gnawing imparted by actidione and sodium silicate.

Barrier A, left. At the top, sodium silicate (0.1 Gm./sq.in.). Beneath, actidione (0.05 Gm./sq.in.).

Barrier B, right. At the top, actidione (0.05 Gm./sq.in.). Beneath, sodium silicate (0.1 Gm./sq.in.).

TABLE I

Barrier No.	No. Rat Used	Total No. Hours Barrier Exposed to Rat Attack	Condition of Barrier at End of This Period	Comments
1	9	86	Penetrated	One rat died twenty-four hours after removal from test cage
2	14	122	Surface sheet removed only	No animals died. One, however, appeared sick on sixth day after exposure
3	8	76	Penetrated	No animals showed signs of being sick
4	8	75	Penetrated	Barrier slipped so one edge was available. It is felt this facilitated penetration
5	2	5	Penetrated	Here again the barrier slipped out of position allowing the animal to attack it along the edge
6	4	53	Only slight damage to surface sheet	None of the animals showed any ill effects from contact with the barrier
7	1	1	Penetrated	This animal attacked barrier near edge and was observed peeling back the laminated sheets. It had no ill effects as a result of contact with barrier
8	4	53	Surface sheet removed only	Animals showed no ill effects
9	4	53	Only slight damage to surface sheet	Animals showed no ill effects
10	4	53	Only slight damage to surface sheet	Animals showed no ill effects

In the tests involving the dual-treated barriers, barrier *A* (Fig. 2) was mounted with the actidione-treated portion at the bottom of the frame, while the sodium silicate portion of barrier *B* was at the bottom. This difference in position did not affect the results, since attacks on both barriers were largely confined to the sodium silicate-treated portions. After one hundred and one hours of continuous exposure to attack, the actidione-treated portion of barrier *A* was damaged only slightly. The actidione-treated portion of barrier *B* showed greater damage after exposure of one hundred ninety-nine hours, but again penetration took place largely through the portion treated with sodium silicate. For a period of days prior to penetration, much of the protective coating of both halves of barrier *B* was gone. Even so, test animals which regularly would gnaw through V-3S fiberboard barriers within one to two hours were reluctant to attack the test barrier. None of the animals used in this test showed any ill effects from contact with the antibiotic.

DISCUSSION

On the basis of these tests, it is evident that actidione is highly repellent to laboratory rats and that barriers containing this compound are highly resistant to gnawing attacks by these animals. The animals were observed to avoid the barriers after initial contact and there was little evidence that any animal reattacked a barrier during subsequent exposure periods. Similarly, starved animals which had initially rejected treated food would again refuse to eat such food on a subsequent exposure.

Penetration of the barriers in a number of instances was facilitated by the barrier slipping out of position so that the rat could attack along one edge. In this way, the sheets of the laminate could be removed with minimum contact with the active material.

It should be pointed out that coating the barrier or plies of a laminate with actidione at a rate of 0.05 Gm./sq. in. was a routine screening procedure and does not constitute a recommendation that this concentration be used in practical application. It should not be implied that this is the minimum rate of application which has a marked repellent effect—that rate is yet to be determined. The high order of repellent activity shown by the acceptance tests might indicate that protection of packaging materials might be achieved through the use of relatively small concentrations of the compound.

Although the toxicity and irritant properties of actidione might limit its use as a rodent deterrent, these studies have shown that protection against rodent attacks may be achieved through the use of relatively small concentrations of an active material. It should be noted that in no case was it certain that an animal died as the result of contact with actidione during the barrier experiments, which may indicate that methods may be devised for the elimination of hazards connected with such use of the compound. However, definite proof of this point must be obtained before the use of actidione as a rodent repellent can be recommended.

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The Spectrophotometric Examination and Acute Toxicity of Rutins Obtained from *Fagopyrum esculentum* (Buckwheat) and *Sophora japonica*[*,†]

By J. W. E. HARRISSON, BELLA W. SELTZER, and ERIC W. MARTIN

The results of comparative toxicity tests on rutins isolated from *Sophora japonica* (*Papilionaceæ*) and *Fagopyrum esculentum* (*Polygonaceæ*) are presented. The acute toxicity of *S. japonica* rutin when administered intravenously to mice does not differ from that of *F. esculentum*. The absorption spectra of the two rutins in the ultraviolet are identical.

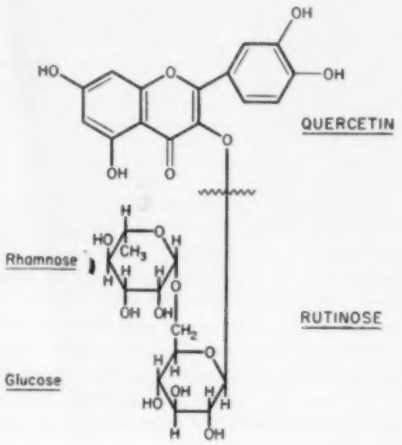
THE flavonoid glycoside rutin was first isolated in 1842 from *Ruta graveolens* (common rue) by Weiss (1). During the next one hundred years it was obtained from approximately 40 species of plants under many names, including rutoside, eldrin (2), melin, phytomelin, myrticolorin, osyritin, and violaquercitrin (3). A comprehensive bibliography of 224 references (4) covering the chemistry, pharmacology, and clinical uses of rutin has been published.

Little interest was evidenced in rutin until Dr. J. F. Couch of Eastern Regional Research Laboratories showed, in collaboration with Dr. J. Q. Griffith (6), that the glycoside possessed "vitamin P" activity (decreases capillary fragility). Couch, Naghski, and Krewson (12) obtained their first supplies of rutin from tobacco, but later developed methods for its extraction from the more commercially practicable source, buckwheat.

The use of this glycoside in capillary fragility and hypertension is now widely accepted (5–8) and it is recognized by the N. F. IX (1950) which states:

"Rutin is the 3-rhamnoglucoside of 5,7,3′,4′-tetrahydroxyflavonol obtained from buckwheat, *Fagopyrum esculentum* (Fam. *Polygonaceæ*) or other sources and contains, on a moisture-free basis, not less than 95 per cent $C_{27}H_{30}O_{16}$."

It may also be described chemically as the 3-rutinoside of quercetin (3,5,7,3′,4′-pentahydroxyflavone), rutinose being 6-D-glucose-β-L-rhamnoside (9). The structural formula is shown at the top of the next column.



Rutin was first isolated by Stein in 1853 from *Sophora japonica* under the name of "melin." It was also obtained from this plant in 1924 by Charaux (10). *S. japonica* is a species of a papilionaceous genus known to contain toxic alkaloids of the lupinane group (11). This source is more familiarly known as the "pagoda tree" and is cultivated by the Chinese for ornamental purposes. The flower buds, sold as "Chinese yellow berries," have been used for many years to dye garments yellow, one of the yellow pigments being rutin. However, this source had not been exploited commercially until recently when it was discovered that *S. japonica* contained several times as much rutin as buckwheat (12, 13). Some analyses run as high as 22 per cent of rutin on the dried basis (14).

The possible presence of toxic principles in *Sophora* species (11) necessitates rigid purity control and advisedly indicates toxicity control. Accordingly, purified samples of *S. japonica* rutin have been compared spectrophotometrically with buckwheat rutin to determine identity and purity. In addition, an acute toxicity study has been made to compare the toxicities of the two rutins.

* Received May 5, 1950, from the Research Laboratories of LaWall and Harrisson, Philadelphia, Pa.
Presented to the Scientific Section, A. PH. A., Atlantic City meeting, May, 1950.
† This investigation was undertaken at the request of the J. T. Baker Chemical Company, Phillipsburg, N. J., and is published with their permission.

ABSORPTION SPECTRA

The ultraviolet absorption spectra of several flavone and flavonol glycosides, including rutin, were compared in 1925 by Tasaki (15). He showed that the character of the absorption spectra was influenced by the position of attachment of the sugar moiety. Therefore, identical spectra, in conjunction with the melting point of the aglycone quercetin, obtained by hydrolysis, are usually considered to be sufficient proof of identity. The percentage of light transmitted at the absorption maxima is an index of the purity of the glycoside and the spectrophotometric method of assay (16) is now officially recognized by the National Formulary.

Experimental

Apparatus.—The absorption spectra reported were determined with a model DU Beckman Spectrophotometer, using 1-cm. quartz cells. The cells were calibrated and all absorbancies reported are corrected.

Rutin, Samples.—Three samples of buckwheat rutin and three samples of *S. japonica* rutin were submitted by the J. T. Baker Chemical Company and were examined as received after a preliminary drying. The analytical data for the six samples are shown in Table I.

Determination of Spectra.—Buckwheat rutin sample No. 3 and *Sophora* rutin sample No. 4 were used for spectrometric comparison. Exactly 50 mg. of each sample as received was dissolved in 100 ml. of hot aldehyde-free alcohol, cooled, and diluted to 100 ml. with the alcohol. A two-milliliter aliquot was then diluted with 1 cc. of 0.02 *N* acetic acid and sufficient alcohol to make 100 ml. (1 mg./100 ml.). The absorbancies for each solution were plotted against the corresponding wave lengths from 220 to 800 mμ.

Discussion

The ultraviolet absorption spectra for both *Sophora japonica* and *Fagopyrum esculentum* rutins are shown in Fig. 1. They are identical except for a very slight variation in absorption at the maxima and minima. This is due to the fact that the curves were determined on the samples as received. If both series of specific absorption coefficient values at the different wave lengths are calculated back to the two rutins on a moisture-free basis and a very small correction is made for quercetin content the two curves are then identical.

The specific absorption coefficient at $\lambda_{362.5}$ for the Reference Rutin of the National Formulary when dried at 125° for sixteen hours over barium oxide under a vacuum of less than 3 mm. of mercury is

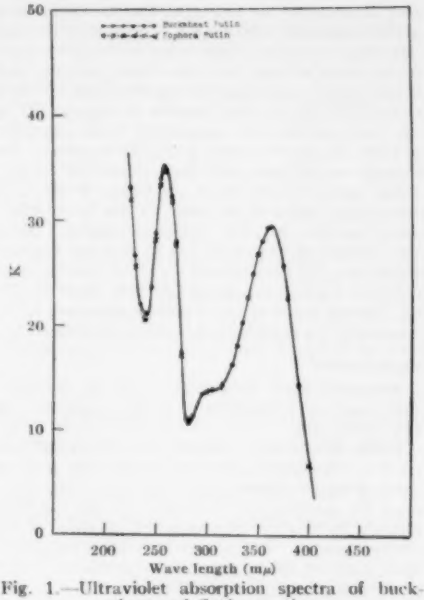


Fig. 1.—Ultraviolet absorption spectra of buckwheat and *Sophora* rutins.

33.22. The lower *K* values indicated by the curves in Fig. 1 are the result largely of moisture content (see Table I).

ACUTE TOXICITY

The toxicity of rutin was first investigated in 1913 by Garino (17) who administered the glycoside orally to dogs. He found it to be practically nontoxic. Other workers (18–21) using different animals also reported rutin to be nontoxic when given orally. There has been very little published, however, on the acute toxicity of the glycoside. Wilson, Mortarotti, and Doxtader (22) of Western Regional Research Laboratories reported in 1947 that intravenous injections in rats, guinea pigs, and rabbits of 30–200 mg./Kg. were innocuous, and they concluded that "on the basis of the criteria employed, rutin is nontoxic both acutely and chronically." In order to compare the relative toxicities of the two rutins it was necessary to administer sufficient of the drug, if possible, to produce some toxic manifestations. Notwithstanding the fact

TABLE I.—ANALYTICAL DATA FOR RUTINS*

	Buckwheat			*Sophora*		
Sample No.:	1	2	3	4	5	6
Moisture	7.43	6.42	5.35	7.53	7.80	7.95
Ash	0.06	0.06	0.02	0.00	0.05	0.04
Rutin	97.90	98.40	96.40	96.26	95.60	96.58
Quercetin	0.85	1.70	2.47	0.57	1.20	0.11
Chlorophyll	0.0005	0.0005	0.0002	0.0000	0.0002	0.0015
Red pigment	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000

* The above calculations, except for water content, are on the anhydrous basis.

that Wilson and his colleagues had used propylene glycol solutions without the development of any untoward symptoms, larger doses were administered in the same solvent, as it was found possible after investigation to increase the concentration of rutin in this solvent. A large number of animals, 177 in all, were injected with increasingly large quantities of rutin in order to obtain a definite response. Although no end point had been established in previous acute toxicity tests, at higher levels both rutins were found to be toxic. There is no difference, however, in their degree of toxicity. Since the amount of propylene glycol injected intravenously was well below the M. L. D. of 16.8 Gm./Kg. reported for rats by Seidenfeld and Hanzlik (23) the solvent *per se* is not therefore presumed to be responsible for the degree of toxicity established.

Experimental

Animals.—Male Swiss albino mice of the Fairfield strain, each weighing 18–25 Gm., were used as the test animals.

Rutin Injections.—The solubility of the rutin in various solvents tried was so meager that suspensions of the glycosides were first used. These were made by grinding the rutin in relatively nontoxic solvents of which propylene glycol appeared to be the most useful. Preliminary tests (data not given) were made by diluting a 40% suspension of rutin in propylene glycol with an equal volume of isotonic sodium chloride solution. Thirty mice were injected in the tail vein with 0.25 ml. of this 20% suspension, five mice being used for each sample of rutin. At the end of twenty-four hours all 15 mice injected with buckwheat rutin were alive, with sample No. 4 one had died and one was very ill, with sample No. 5 one was ill, and with sample No. 6 two had died, the last three samples being *Sophora* rutin. Experiments were then set up with controls, using a rutin suspension. The results are recorded in Table II.

It was felt that many of the deaths observed might be due to the intravenous injection of suspended material, and a near approach to the possible M. L. D. for the propylene glycol. Furthermore there was the distinct possibility that the actual toxicity of the rutin was unmanifested by reason of its not being in solution. To eliminate these questionable points many attempts were made to prepare rutin solutions of sufficient concentration whereby an adequate dose could be given within the capacity of the animal and without any likely side effect by the solvent. It was eventually found that solution of the rutin could be effected by warming a 20% suspension of the glycoside in propylene glycol to about 75°. The greenish, slightly turbid solutions were cooled to 37° before administration. Prolonged heating was avoided because otherwise a change in color took place, indicating that possibly alteration of the rutin through hydrolysis or other degradative reactions was taking place Prolonged standing was also avoided because the rutin comes out of solution as a thick unctuous mass if allowed to stand at room temperature.

The data recorded in Table III are the results obtained with these injections of rutin solutions, and they are to be distinguished from data on the injection of rutin suspensions.

Discussion

Using suspensions of the two rutins, *Sophora* and buckwheat, it was noted that the LD_{50} dose was probably above 3500 mg./Kg. of mouse. Subsequent tests indicated that the LD_{50} was likely below 5000 mg./Kg. (Table II). It was felt that these results accentuated at least two of three possible variables, i.e., suspended solids, lack of complete solution of the rutin, and near approach to the M. L. D. for the co-solvent propylene glycol. Further work was confined to bringing the rutin into solution and reducing the amount of propylene glycol injected.

TABLE II.—ACUTE TOXICITY OF RUTIN SUSPENSIONS[a]

Sample	Approximate Dose of Rutin,[b] Mg./Kg.	No. Mice	Mortality at Days 1	2	3	4	5–14	Total Mortality
Solvent								
Propylene glycol and Normal salt solution aa	None	10	1	..	..	..	..	1/10
Buckwheat rutin								
No. 1	3500	5	..	2	..	..	..	2/5
	5000	3	1	1	1	..	..	3/3
No. 2	3500	5	..	..	1	..	..	1/5
	5000	3	1	..	1	..	..	2/3
No. 3	3500	5	..	1	..	..	..	1/5
	5000	3	2	..	..	..	..	2/3
Sophora rutin								
No. 4	3500	10	2	1	1	..	..	4/10
	5000	3	1	2	..	..	..	3/3
No. 5	3500	5	..	..	1	..	..	1/5
	5000	3	1	1	1	..	..	3/3
No. 6	3500	5	2	..	..	..	..	2/5
	5000	3	1	..	..	..	..	1/3

[a] Dose: 0.5 cc. of rutin suspension in equal parts propylene glycol and normal salt solution per 18–25-Gm. mouse, by tail vein and observed for fourteen days.

[b] 3500-mg. doses given in 14.3% suspension; 5000-mg. doses given in 20% suspension. These doses were based on an average rat weight of 21.5 Gm. The actual dosage varied from 3250 mg./Kg. of mouse to 3650 mg./Kg. of mouse for the 14.3% suspension and from 4840 mg./Kg. of mouse to 5270 mg./Kg. of mouse for the 20% suspension.

TABLE III.—ACUTE TOXICITY OF RUTIN SOLUTION[a]

Sample	Approximate Dose of Rutin, Mg./Kg.	No. Mice	Mortality at Days 1	2	3	4	5-14	Total Mortality
Propylene glycol	None	10			None			0/10
Buckwheat rutin								
No. 1	940	10			None			0/10
No. 2	940	10	3	..	..	..	2	5/10
No. 3	940	10	..	..	..	..	1	1/10
Total mortality								6/30 (20%)
Sophora rutin								
No. 4	980	10	..	..	..	..	1	1/10
No. 5	980	10	1	1	..	1	1	4/10
No. 6	980	10	..	..	..	1	2	3/10
Total mortality								8/30 (26%)

[a] Dose: 0.1 cc. of 20% rutin solution in propylene glycol, per 18-25-Gm. mouse, given by tail vein and observed for fourteen days.

The solution of the rutin was affected as previously described whereupon preliminary testing of this solution was undertaken to orient its toxic level. Final data upon injections given at levels of 940 and 980 mg./Kg. of mouse are reported in Table III. Based upon these data the buckwheat and *Sophora* rutin show no differences as to over-all acute toxicity and the apparent LD_{50} is slightly greater than 900 mg./Kg. of mouse. This figure represents approximately five times the toxicity evidenced by suspensions of the rutin.

In evaluating these data which indicate a toxic level for rutin, consideration must be given to the method found necessary to administer the drug. Furthermore, there is little likelihood that these findings are significant under therapeutic application where administration is either by the oral route or employing solubilized (modified) forms of rutin by the parenteral route. The most significant finding is that the rutins from these sources show no difference in toxicity.

SUMMARY

1. Comparative ultraviolet absorption spectra are presented for rutins obtained from *Fagopyrum esculentum* (buckwheat) and *Sophora japonica*.

2. An investigation has been made of the comparative acute toxicities of the rutins obtained from the above two sources, the LD_{50} for each being about 950 mg./Kg. of mouse by the intravenous route when in a propylene glycol solution and about 4000 mg./Kg. when in suspension. These toxicity data are comparative only and do not necessarily reflect any suspicion of toxicity under conditions of therapeutic use.

3. The two rutins are identical with respect to both their ultraviolet absorption spectra and their relative toxicities.

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Experiments with the Pressure Cooker in Drug Extraction*,†

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Studies of the individual drug extractions were made, and it was concluded that **nux vomica tincture, belladonna tincture, hyoscyamus tincture, stramonium tincture, cascara sagrada fluidextract, and wild cherry syrup could be made successfully *by the pressure cooker method of extraction. Ipecac fluidextract, ginger fluid*extract, and nux vomica fluidextract which would conform to the official standards could not be made by this method. The principal advantage of this method is the *fact that there is a considerable saving of time. It was concluded that by the pressure cooker method, tinctures of official quality could be made from the alkaloidal* drugs, especially those of the *Solonaceae* family.**

FEW IDEAS pertaining to new methods of extraction of vegetable drugs have been advanced in recent years. Bay and Gisvold (1) and King and Gisvold (2) have performed extraction studies on digitalis, using the Waring blendor. Most of the work which was done on extraction had as its objective the improvement of the extraction methods which have been used for many years, the ultimate goal being to obtain quicker and less tedious methods of extraction.

It seemed logical to assume that, since time could be saved in the cooking of fresh vegetable matter by the use of the pressure cooker, time could also be saved in the extraction of the desired principles of dried vegetable matter (drugs), if the dried material were macerated with the proper menstruum before cooking. It was believed that the breaking down of the plant tissues by cooking under pressure would make possible the extraction of the active principles in a shorter period of time than that required for extraction by the methods now in general use. However, there was some doubt as to the feasibility of the method, because many of the active principles of drugs are heat labile and might undergo change or decomposition in the process of cooking. Notwithstanding this possible shortcoming, the practicability of the method seemed worthy of investigation.

The following official preparations were studied: nux vomica tincture, belladonna tincture, hyoscyamus tincture, stramonium tincture, nux vomica fluidextract, ginger fluidextract, ipecac fluidextract, cascara sagrada fluidextract, and wild cherry syrup.

EXPERIMENTAL AND RESULTS

The preparations were made by the official methods and by the pressure cooker method and were compared with respect to specific gravity, *p*H value, color of the freshly prepared product, appearance of the preparation after storage for six months, odor, taste, total extractive, and active principle content.

With the exception of the comparisons of the active principle contents, all comparisons were made after each of the preparations was adjusted to the same active principle content, the average of the official monographs being taken as the point of adjustment. In the case of nux vomica tincture No. 5, where the value is slightly below the average stated in the monograph, the tincture made by the official method was adjusted to the same strychnine content as nux vomica tincture no. 5. This procedure was not applied to the fluidextracts or wild cherry syrup.

The Mirromatic[1] pressure cooker, 4-quart capacity, was used in these experiments. Its pressure range is 5, 10, and 15 pounds per square inch.

Method of Operation of the Cooker

The drug was weighed, placed in the pressure cooker, and after the addition of a calculated amount of menstruum, was macerated for a specified period of time in the closed cooker. The pressure control was placed over the vent tube at the number 15 hole, indicating that 15 was the number of pounds of pressure desired. The cooker was then placed on a tripod over a Bunsen burner with a high flame. When the desired pressure was reached, the pressure control jiggled and sputtered constantly. The cooking time was counted from this moment. The flame was reduced so that the control jiggled only about three times per minute. After the desired pressure had been maintained for the prescribed time, the cooker was removed from the tripod and placed under a stream of cold water to reduce the

* Received May 5, 1950, from the College of Pharmacy, George Washington University, Washington, D. C.
Presented to the Scientific Section, A. PH. A., Atlantic City meeting, May, 1950.
A portion of a thesis submitted as partial fulfillment of the requirements for the degree of Doctor of Philosophy, School of Pharmacy, University of Maryland.
† This work was made possible by the award of a fellowship to S. J. Greco by The American Foundation for Pharmaceutical Education.
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[1] Manufactured by Aluminum Goods Manufacturing Co., Manitowoc, Wis.

pressure. After the pressure registered zero, the cooker was opened and the material was filtered *through cotton in an ordinary glass funnel. When* the filtrate began to come through clear, it was returned to the funnel, and the filtration was continued until an amount equal to that specified in the U. S. P. XIII or N. F. VIII for assayed tinctures or fluidextracts had been collected. If necessary, *a sufficient quantity of menstruum was added through the filter to produce that specified quantity.* Upon different occasions, modifications of the above procedure were performed. They will be noted throughout the discussion.

All of the official tinctures were made by Process P (3), whereas the fluidextracts were made by Process *A (4), B (5), or D (6).* The products made by the pressure cooker method had the same ratio of crude drug to the finished volume as their official counterparts.

Physical Constants and Properties of the Preparations

Physical Properties.—The physical properties studied included odor, taste, color of the freshly prepared product, and appearance after storage for six months. These values appear in Table I.

Specific Gravity.—This constant was determined with a Mohr-Westphal balance at 25°, and the values are reported in Table II.

pH Values.—These were determined with a Beckman pH meter and are reported in Table II.

Determination of Total Extractive

The total extractive of the preparations was determined in the following manner:

Measure accurately 50 cc. of the preparation into a tared beaker by means of a pipette, the beaker having been previously heated to constant weight in an oven at 110°. Evaporate the sample to dryness on a steam bath and dry to constant weight at 110°. These values are shown in Table III.

TABLE II.—SPECIFIC GRAVITIES AND pH VALUES OF THE PREPARATIONS

Preparations	Specific Gravity at 25°		pH Value	
	Official Method	Cooker Method	Official Method	Cooker Method
Nux vomica tincture	0.887	0.900[a]	1.70	5.15[a]
Belladonna tincture	0.884	0.895	6.27	6.32
Hyoscyamus tincture	*0.888*	*0.899*	*6.05*	*6.12*
Stramonium tincture	0.885	0.925	6.17	6.17
Ginger fluidextract	0.855	0.861	6.50	6.67
Ipecac fluidextract	1.012	0.972	0.63	0.77
Cascara sagrada fluidextract	1.078	1.083	5.00	4.95
Wild cherry syrup	1.286	1.282	4.65	4.65

[a] Nux vomica tincture No. 5 was used in the above comparisons.

Assays

All of the preparations were assayed by the official methods. These values are shown in Table III.

Extraction of the Specific Drugs

Nux Vomica.—Five nux vomica tinctures were prepared.

Nux vomica tincture No. 1 was prepared by the official method.

Nux vomica tincture No. 2 was prepared from the following formula:

Nux vomica, in moderately coarse powder	40 Gm.
Hydrochloric acid	3 cc.
Alcohol	285 cc.
Water	92 cc.

TABLE I.—PHYSICAL PROPERTIES: COLOR, ODOR, TASTE, AND CHANGES ON STORAGE OF THE PREPARATIONS

Preparations	Color		Odor		Taste		Changes on Storage	
	Official Method	Cooker Method	Official Method	Cooker Method	Official Method	Cooker Method	Official Method	Cooker Method
Nux vomica tincture	Orange	Yellow[a]	Winelike	Winelike	Very bitter	Very bitter	From orange to reddish orange	From yellow to amber
Belladonna tincture	Dark greenish brown	Dark greenish brown	Tobacco-like	Tobacco-like	Alcoholic	Alcoholic	No apparent change	No apparent change
Hyoscyamus tincture	Dark greenish brown	Dark greenish brown	Tobacco-like	Tobacco-like	Slightly pungent	Slightly pungent	No apparent change	No apparent change
Stramonium tincture	Dark greenish brown	Medium brown	Slightly tobacco-like	Slightly tobacco-like	Pungent	Slightly pungent	Slight precipitate	No apparent change
Ginger fluidextract	Deep reddish brown	Deep reddish brown	Spicy	Spicy	Strongly pungent	Strongly pungent	No apparent change	No apparent change
Ipecac fluidextract	Deep reddish brown	Deep reddish brown	Winelike	Winelike	Burning-sour	Burning-sour	No apparent change	No apparent change
Cascara sagrada fluidextract	Dark brown	Dark brown	Slightly alcoholic	Alcoholic and pungent	Very bitter	Very bitter	Slight precipitate	Slight precipitate
Wild cherry syrup	Medium red	Light red	Cherry-like	Cherry-like	Sweet	Sweet	No apparent change	No apparent change

[a] Nux vomica tincture No. 5 was used in the above comparisons.

TABLE III.—TOTAL EXTRACTIVE AND ALKALOIDAL CONTENT OF THE PREPARATIONS

Preparations	Total Extractive, Percolation Method, Gm./100 Cc.	Alkaloids, Percolation Method, Gm./100 Cc.	Total Extractive, Cooker Method, Gm./100 Cc.	Alkaloids, Cooker Method, Gm./100 Cc.
Nux vomica tincture No. 1	1.3776	Strychnine, 0.1252 0.1241	...	...
Nux vomica tincture No. 2	...	...	...	Strychnine, 0.1133
Nux vomica tincture No. 3	...	...	...	Strychnine, 0.1167
Nux vomica tincture No. 4	...	...	...	Strychnine, 0.0855
Nux vomica tincture No. 5	...	...	1.3050	Strychnine, 0.1110 0.1101
Nux vomica fluidextract	...	Strychnine, 1.264 1.241	...	Strychnine, 0.3912 0.3922
Belladonna tincture	1.1394	0.0503 0.0499	0.9992	0.0503 0.0499
Hyoscyamus tincture	1.7896	0.00495 0.00488	1.4850	0.00519 0.00526
Stramonium tincture	1.5686	0.0427 0.0425	1.5282	0.0361 0.0353
Ginger fluidextract	6.6975 ether-soluble	...	2.8290 ether-soluble	...
Ipecac fluidextract	15.2982	1.9920	10.3712	1.2500
Cascara sagrada fluidextract	24.0886	...	23.5982	...

The nux vomica was allowed to macerate for twenty-four hours and was then cooked for five minutes at 15 lb. pressure. The cooker was cooled, and the tincture was filtered through cotton. Enough of a mixture of 3 volumes of alcohol and 1 volume of water was passed through the filter to make the final product measure 380 cc. The tincture was defatted by cooling for thirty minutes at 5° with subsequent filtration. The tincture thus prepared could not be clarified either before or after defatting. The official assay showed 0.1133 Gm. of strychnine per 100 cc.

The same formula was used for making nux vomica tincture No. 3. In this case, the nux vomica was macerated for only fifteen minutes and then cooked for ten minutes at 15 lb. pressure. This tincture also could not be clarified. The assay showed 0.1167 Gm. of strychnine per 100 cc.

Based on the results obtained from the assays of the two preceding tinctures, the twenty-four-hour maceration period showed no advantage over the fifteen-minute maceration period. Consequently, the twenty-four-hour maceration period was used sparingly throughout the remainder of the experiments.

Nux vomica tincture No. 4 was made from the following formula:

Nux vomica, in moderately coarse powder	40 Gm.
Acetic acid	5 cc.
Alcohol	325 cc.
Water	105 cc.

A maceration period of fifteen minutes was allowed, after which the mixture was cooked for five minutes at 15 lb. pressure. After defatting, the tincture obtained was perfectly clear. The assay showed only 0.0855 Gm. of strychnine per 100 cc.

Since, in the foregoing experiment, a low yield of strychnine was obtained, another experiment was run to determine if an increase in the length of the cooking period would yield a greater amount of strychnine.

Nux vomica tincture No. 5 was made in the same manner as the above tincture, with the exception that in this case the mixture was cooked for ten minutes at 15 lb. pressure. The tincture obtained in this case was also clear. The assay showed 0.1110 Gm. of strychnine per 100 cc. Two hours were required to make this preparation, exclusive of the defatting period.

In view of these results, it was decided to make all of the remaining tinctures by the use of a fifteen-minute maceration period, followed by cooking for ten minutes at 15 lb. pressure.

Two nux vomica fluidextracts were prepared, one by the official method and the other by the pressure cooker method. The following formula was used in the pressure cooker method:

Nux vomica, in moderately coarse powder	400 Gm.
Acetic acid	40 cc.
Water	185 cc.
Alcohol	675 cc.

The mixture was allowed to macerate for twenty-four hours and was then cooked for five minutes at 15 lb. pressure. The assay showed only 0.3912 Gm. of strychnine per 100 cc. This may readily be understood, inasmuch as the official procedure directs that the nux vomica be exhausted, whereas in the above formula, only the first 340 cc. of fluid-

extract was collected, and a portion of that was assayed. In view of the low results, no further experiments were conducted along this line.

Belladonna Leaf, Hyoscyamus, and Stramonium.—Tinctures of these drugs were made according to the following formula:

Crude drug, in fine powder	40 Gm.
Alcohol	325 cc.
Water	110 cc.

The mixture was allowed to macerate for fifteen minutes and was then cooked for ten minutes at 15 lb. pressure. The mixture was filtered through cotton, and a sufficient amount of a mixture of 3 volumes of alcohol and 1 volume of water was passed through the filter to make the filtrate measure 380 cc. Three hours were required to make each of these preparations.

The results of the assays showed that the tinctures were comparable to those made by the official method. However, this did not necessarily prove that hydrolysis did not occur. Atropine and hyoscyamine are the principal alkaloids of belladonna tincture. If these alkaloids were hydrolyzed, tropanol would be formed. If tropanol were present, the same amount of 0.02 *N* acid would be required to react with 1 mole of it as with 1 mole of the alkaloids of belladonna leaf. Consequently, if hydrolysis did occur, it could not be detected by the titration. Therefore, the alkaloids of belladonna tincture, made by the pressure cooker method, were isolated and the picrate was made. The official belladonna tincture was treated in a similar manner. The melting point of the picrate made from the pressure cooker tincture was 168–170°. The melting point of the picrate made from the official tincture was 164–166°. The reported melting point of atropine picrate is 175–176° (7), and that of hyoscyamine picrate is 165° (8). However, tropanol picrate melts with decomposition at 275° (9). Therefore, it was concluded that no hydrolysis of the alkaloids of belladonna tincture occurred during the preparation of the tincture by the pressure cooker method. Since the alkaloids of hyoscyamus and stramonium are similar in chemical structure, the same may be said of the tinctures of these drugs made by the pressure cooker method.

Ginger.—The formula used in the preparation of ginger fluidextract is as follows:

Ginger, in fine powder	200 Gm.
Alcohol	540 cc.
Water	60 cc.

The mixture was allowed to macerate for fifteen minutes and was then cooked for ten minutes at 15 lb. pressure. The mixture was filtered through cotton, and enough of a mixture of 9 volumes of alcohol and 1 volume of water was passed through the filter to make the filtrate measure 170 cc.

Ipecac.—The formula used in the preparation of ipecac fluidextract is as follows:

Ipecac, in fine powder	100 Gm.
Alcohol	300 cc.
Water	100 cc.

The mixture was allowed to stand overnight and was then cooked for ten minutes at 15 lb. pressure. After filtration, the filtrate was evaporated to 100 cc., and 200 cc. of water was added to it. The mixture was allowed to stand overnight and filtered. The filtrate was then evaporated to 56.5 cc., and 3.5 cc. of hydrochloric acid and 30 cc. of alcohol were added to the evaporated filtrate.

Cascara Sagrada.—The formula used in the preparation of cascara sagrada fluidextract is as follows:

Cascara sagrada, in very coarse powder	100 Gm.
Boiling water, a sufficient quantity	
Alcohol	20 cc.

The cascara sagrada was placed in the pressure cooker, and 400 cc. of boiling water was poured over the drug. The mixture was allowed to macerate for fifteen minutes and was cooked for ten minutes at 15 lb. pressure. The material was filtered through cotton, and boiling water was passed through the drug until it was exhausted. The filtrate was evaporated to 80 cc. on a water bath and allowed to stand in a stoppered container for several days. The clear liquid was decanted and the remainder was filtered into the decanted liquid. The residue on the filter was washed with a sufficient quantity of a mixture of alcohol and water to make the fluidextract measure 100 cc. and contain the required proportion of alcohol. Two hours were saved in the manufacture of this preparation, when compared to the time required to make the official fluidextract.

It should be noted that the drug in this case was completely exhausted. This was not true in the case of the methods used for nux vomica, ginger, and ipecac fluidextracts.

Wild Cherry.—Wild cherry syrup was made according to the following formula:

Wild cherry, in coarse powder	30 Gm.
Glycerin	30 cc.
Sucrose	135 Gm.
Alcohol	4 cc.
Water, a sufficient quantity, To make	200 cc.

The wild cherry was placed in the cooker, and 130 cc. of water was poured over the drug. The mixture was allowed to macerate for fifteen minutes and then was cooked for five minutes at 15 lb. pressure. After filtration, sufficient water was passed through the filter to make the total volume of the filtrate measure 80 cc. The filtrate was filtered until clear and added to the sucrose, which was dissolved by agitation. Then the glycerin, alcohol, and sufficient water were added to make the final product measure 200 cc. Only one hour was saved in the manufacture of this preparation.

Because of the fact that there are no official assays for cascara sagrada fluidextract and wild cherry syrup, comparisons could only be made with respect to physical properties, pH value, and specific gravity. Total extractive was also determined for cascara sagrada fluidextract.

DISCUSSION

It may be said, in general, that there is very little difference in the physical properties of the preparations made by the pressure cooker method as compared with those made by the official method. The one exception is in the case of the two nux vomica tinctures, in which there is a difference in color. This is thought to be due to the fact that there is less total extractive in the tincture prepared by the pressure cooker method.

The *p*H values and specific gravities of the preparations made from the same drug checked very closely. Again the exception is in the nux vomica tinctures, in which case the *p*H values are considerably different. This difference is readily understandable when it is recalled that hydrochloric acid was used in the preparation of the tincture by the official method, whereas acetic acid was used in the preparation of the pressure cooker tincture.

The total extractive was found to be less in the pressure cooker preparations than that found in the preparations made by the official methods. This may be explained on the basis of the slow rate of solubility of some of the constituents of the extractive or on the assumption that some of the constituents, such as albumin and proteins, which are present in plants and which are ordinarily found in the extractive, are coagulated and rendered insoluble by the application of heat which is required in the pressure cooker method. The fact that there is less total extractive obtained by the cooker method is one of the important advantages of using this method, since it can be said, as a general rule, that the less the extractive content of a preparation, the more stable it is likely to be.

In those cases where the cooker, without subsequent complete exhaustion of the drug, gave unsatisfactory results, for example in the preparation of fluidextracts, it is believed to be due to the comparatively small amount of menstruum per weight of drug as compared to the amount of menstruum per weight of drug in the preparation of tinctures. Therefore, the quantity of menstruum used was not sufficient to exhaust the drug of its active principles.

SUMMARY AND CONCLUSIONS

1. Several official galenicals involving the process of percolation were prepared by the use of the pressure cooker and were compared with similar products made by the official methods.

2. The pressure cooker method was found to be efficient for the preparation of tinctures made by extraction procedures.

3. A satisfactory modification of the official formula for making nux vomica tincture by the pressure cooker method was devised.

4. With respect to physical properties, it appears that the cascara sagrada fluidextract and wild cherry syrup, prepared by the cooker method, were equally as good as those made by the official methods.

5. In general, fluidextracts of official quality could not be prepared by the cooker method; however, the method as used in the preparation of cascara sagrada fluidextract gave a satisfactory product. Further work is necessary to determine if other fluidextracts can be efficiently and satisfactorily made by this same method.

6. In the cases where the cooker method is satisfactory, it has many advantages over the official extraction methods. There is, in most instances, a considerable saving of time. Packing of the percolator and regulation of the flow of percolate are eliminated. All of the preparations made by this method contain less total extractive than their official counterparts, and the less total extractive, the more stable the finished product is likely to be.

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Study of Odors*

By N. N. TANYOLAC and J. R. EATON

Odors have been difficult to measure and classify with accuracy principally because no objective measuring method has been developed. The mechanism of olfaction is but little understood; several theories have been presented but none has been generally accepted. This paper describes experiments in which an attempt has been made to detect contamination of the air at concentration recognized by the nose as strong odors. Studies have been made on the change of surface tension of a liquid drop due to air contamination resulting from nearby solid odorous material. The experiments described herein indicate that the surface tension of distilled water, mineral oil, and water-stabilized mercury may undergo considerable change when the air in contact with the drop is contaminated. The change of surface tension of a particular liquid follows a pattern characteristic of the contaminant. The same contaminant produces different surface tension changes in the three different liquids studied. The results of these tests suggest that an instrument might be developed which would be capable of classifying and measuring air contamination at low concentrations.

MAN experiences the outside world through his senses, which are usually classified as sight, hearing, touch, taste, and smell. In addition to these, there are the more obscure senses less commonly recognized. All communications between an individual and his surroundings are by means of one or more of these senses. The relative importance of these several senses varies greatly between different animals, each developing through training and evolution those senses which serve him best.

It is of interest to observe that a large segment of scientific development stems from the fact that man has been able to develop instruments which extend the range and acuity of his several senses. This fact is obvious when we consider the importance of the microscope, the telescope, the spectroscope, and other instruments with which we are able to study light energy in a range far beyond that of the unaided eye. The science of acoustics was developed to a considerable extent from our ability to study sound waves by means of instruments. The development of distance and weight measuring instruments is, in fact, an extension of our sense of touch through instruments. The senses of taste and smell have been slow to develop, with the result that man possesses no significant aids in these areas.

The importance of the sense of smell, even with our relatively ineffective nose, is apparent in our selection of suitable foods and, in a more esthetic sense, in our selection and development of perfumes. The chemist uses the sense of smell to a limited extent in his identification of chemicals (1), while a physician uses the sense of smell to about the same degree in the identification of diseases (2, 3). The use of the sense of smell in the fields mentioned above has not been developed to the point where the scientist relies heavily upon his nose for decisions of significance in his work.

Although civilized man uses the sense of smell in a secondary manner, it is well known that certain primitive tribesmen are able to use this sense much more effectively. In the case of the lower animals, the sense of smell is frequently of more importance than those senses on which man relies most highly. Dogs use the sense of smell in following trails, while other animals use this sense in searching out food and in avoiding danger.

It is interesting to contemplate the significance of a device which would combine the acuity of the nose of a lower animal with the reasoning power of the human mind. It is possible that such a development might put the sense of smell on a level with the sense of sight. Such a development would, without doubt, be of considerable importance in such fields as chemical analysis, medicine, identification of individuals, and perhaps in other fields not at present anticipated.

THEORETICAL CONSIDERATIONS

Numerous theories of the mechanism of the sense of smell have been proposed by various investigators. It is of interest that the investigational work in this area, while started centuries ago, has not attracted the attention of the modern scientist to any great extent until the past year or so. Many of the more serious investigators who worked in this area a number of years ago were without the aid of modern scientific apparatus (4, 5). In recent years studies in this field have increased, though for sometime they were carried on principally by individuals interested in odors from the standpoint of perfume manufacturing, coffee blending, and simi-

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lar pursuits (6). More recently substantial contributions have been made in this area of investigation by individuals working on the subject from the purely scientific approach (5, 7). The theories of the mechanism of olfaction may be summarized briefly as follows:

1. Vibration.—Inasmuch as the sense of sight is dependent on electromagnetic vibration, and the sense of hearing is dependent on material vibration, it has been reasoned that perhaps odors are transmitted by a vibration of some sort. Although this analogy is quite logical, there has been no substantial scientific proof of the existence of vibrations which carry odors. Some investigators have proposed that the odor of materials is due to the vibration of the electrons within the molecules of the odorous materials which are drawn into the nose.

2. Chemical Structure and Vibration.—According to this theory, the type of odor depends on the presence of odoriphores (special chemical groups responsible for the odor), and the sense of smell is considered to be due to molecular vibration. Studies have failed to establish or to disprove this theory (5).

3. Chemical Reaction.—It is proposed by some that the mechanism of olfaction is due to the chemical reaction occurring between the molecules of material drawn into the nose and the complicated mechanism of the olfactory region of the nose. According to this theory the sensation is possible only when the molecules of the odorous material are soluble in the lipoid fluid of the olfactory region. Experimental studies have failed to establish or to completely disprove this theory (5).

4. Physical.—It has been proposed that the physical action of the molecules of odorous material in contact with the linings of the olfactory region of the nose produced, in some manner, nerve stimulation. Those who propose this theory feel that the variety in odor is due to differences in dipole moment of molecules (5).

5. Infrared Theory.—More recently it has been proposed that olfaction is associated with the infrared absorption of odorant molecules present in the nose in close proximity to the olfactory surfaces. This theory is perhaps attracting most attention at the present time (8, 9).

With the exception of the vibration theory, all other explanations of the mechanism of olfaction require that particles of odorous materials enter the nose and come in contact with, or come in close proximity to, the olfactory area. The exact methods by which these molecules produce nerve stimulation is at the present time obscure and may not be explained in the near future. Of importance in any of the methods proposed is the small amount of odorous material which will excite the sense of smell. It has been demonstrated that many substances are detected by the human nose in concentrations less than 1×10^{11} molecules per liter of air. When it is remembered that air drawn into the nose ordinarily by-passes the olfactory region and enters it only through eddies in the air stream, and when it is remembered that not all of the molecules entering the olfactory cleft can be effective in producing olfaction, it is apparent that the amount of odorous material necessary to stimulate the sense of smell is very small.

ODOR-SENSITIVE DEVICES

As far as is indicated by the present published literature (5, 7), no device has been developed for studying odors directly except instruments which depend on the human nose as the primary indicator. This means then that all investigations of this sort are limited by the many physical and psychological vagaries of the human being and by the inherent insensitiveness of the human nose as compared to that of the lower animal. It is perhaps of importance to note that a few devices have been developed which detect specific contaminations in the air (such as the halogens) and that these devices apparently respond to the groups of materials even though the odors of the materials in the group may be quite different (10, 11).

In attempting to set up experiments by which odors might be detected, it is of importance to try to define *odor*. Immediately this raises several very important problems, for it appears that many materials which are inodorous to the human nose are easily detected by the nose of the lower animals. It is, of course, quite impossible to state whether this difference lies in sensitivity of detection or range of detection. It should perhaps be borne in mind that just as the human eye is blind to light of certain frequencies, which in some cases other animals can detect, and the human ear is deaf to certain frequencies, so the human nose may be entirely insensitive to certain materials which other animals might find odorous. In view of the uncertainties in regard to the limitations of the human olfactory system, it might be to an advantage from the scientific point of view to re-define odors in a fashion far more general than now considered. Perhaps the detection of odors should be defined as the detection of air contaminations of small magnitude regardless of the nature of the contaminant. Such a definition would appear to be in line with our present concept of olfaction if we assume that the nose is sensitive only to certain specific contaminations. This definition would perhaps make all substances odorous, for all materials, both solids and liquids, give off molecules of their substance continually. By this definition, an odor-sensitive device would be one which will detect and identify the presence of submicroscopic contamination in air regardless of the contaminating material concerned. By the above definition it is apparent that water vapor and other materials present in the air but not detected by the human nose would be classed as odorous.

Methods of Detecting Small Concentration of Contaminants.—In searching for a method of detecting odors in accordance with the above definition of odors, it is logical to examine those scientific phenomena in which microscopic causes are known to produce gross results. Several such phenomena are as follows:

1. It is well known that the breakdown of an air gap in the presence of voltage stress is dependent upon the production of a limited number of free electrons from outside sources. Without the incidence of electrons in the gap region as a result of cosmic rays, radioactive material, and other sources, the breakdown of gaps would be a phenomenon quite different from that which is ordinarily observed. This difference is evident in the random

time lag of short gaps subjected to steep wave-front impulses.

2. The emission of electrons from heated surfaces is dependent, to a great extent, on the work function of the surface material. It is well known that minute contamination of the surface of heated electrodes may significantly increase or decrease electron emission. A similar phenomenon relating to positive ion emission is being used at the present time in a device to detect halogen vapor in air.

3. Many chemical reactions are dependent on the presence in the reactor of certain well-known catalysts. Although the catalysts are in many cases present in considerable amount, little or no catalyst is expended in the reaction.

4. The effect on the surface tension of liquids by minute contamination is of particular interest. Studies of surface tension of liquid-to-air surfaces are always plagued by the difficulties which arise from unwanted contamination. The literature on the subject of surface tension has many references to precautions which must be taken in order that the surface will not be contaminated by the breath of the operator or from other sources. The amount of material necessary to produce measurable changes in surface tension is, of course, very small (12–15).

EXPERIMENTAL

Experiments on Surface Tension.—Knowing that small amounts of material may produce measurable changes in surface tension, a series of studies were undertaken to see if odorous materials in reasonable concentrations in the air would also produce measurable changes in surface tension.

In order that a determination could be made of the surface tension of a liquid over an extended period of time without disturbing the liquid, the so-called Pendant Drop Method of surface tension was employed (16, 17).

The Pendant Drop Method consists of suspending a small drop of the liquid under test from the end of a vertical tube. The drop is then photographed by means of a camera and the maximum and the selected diameter of the drop is determined by measurements from the photograph (Fig. 5). The surface tension may be calculated from measured dimensions by the relation

$$\gamma = ge(d_e)^2 \frac{1}{H} \qquad (1)$$

where

γ = surface tension in dynes/cm.
g = acceleration of gravity
e = difference in density between two fluids
d_e = diameter of the drop at equator in cm.
d_s = diameter of the drop in cm. at a plane whose distance from the end of the drop is equal to the equatorial diameter
$1/H$ = a function of the ratio $S = d_s/d_e$ as given in Table I

In Eq. (1) the value of $1/H$ in terms of $S = d_s/d_e$ has been found experimentally by Andreas, Hauser, and Tucker using different sizes of drops of known surface tension value at an age of ten seconds. All the calculations in this study of odor were made by using Eq. (1) and Table I.

Sample of Calculation.—Surface tension of water to air at 25° and at t = six minutes.

$e = d_{water} - d_{air}$
$= 0.99707 - 0.001 = 0.99607$ Gm./cm.^{3}
d_e = 0.453 cm. from 11.48/25.4 where 25.4 units is the magnification and 11.48 units is the measured value of d_e
d_s = 10.88 units in cross-section scale
S = 10.88/11.48 = 0.948 from Table I. $1/H$ is 0.357
$\gamma = ge(d_e^2/H) = (980)\ (0.99607)\ (0.453)^2\ (0.357) = 71.6$ dynes/cm.

Measurements of this type were made with the air as pure as was conveniently possible and then repeated in the presence of solid odorous materials in the immediate vicinity of the liquid surface. No material was added directly to the liquid. The equipment used for this experiment is shown diagrammatically in Fig. 1. A drop of liquid was formed in the chamber at *2*. The drop, illuminated by a light source *1*, was photographed by means of the camera *3*. Subsequent measurement of the photographic records made possible the determination of the surface tension of the liquid drop without disturbing the drop surface. By this method it was possible to take repeated measurements of the surface tension of the liquid as frequently as desired. A photograph of the equipment used is shown in Fig. 2. As may be noted from Fig. 1, the liquid drop was enclosed in a chamber which protected it from air drafts in the room and into which was introduced any odorous substance to be studied. As might be expected, it was necessary to clean the box after each experiment. In order to facilitate cleaning, the box was constructed for convenient disassembly as is shown in Fig. 3. The formation of the drop was controlled by various droppers. One was fed by gravity and was controlled by a stopcock as shown in Fig. 4, *1*. Another dropper, in which the drop formation is controlled by a threaded plunger, is shown in Fig. 4, *2*. A photographic record of a distilled water drop is shown in Fig. 5.

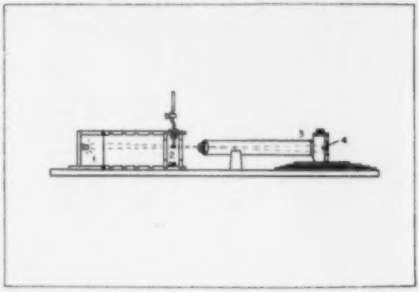


Fig. 1.—Instrument for measuring change of surface tension.
1, light source; *2*, chamber containing suspended drop and solid odorous material; *3*, camera; *4*, calibrating grid in front of film.

Before attempting to study the effects of odors, tests were made to determine the stability of the surface tension of a drop of distilled water in an uncontaminated atmosphere. Figure 6 shows a plot

TABLE I.—ARITHMETICAL TABULATION OF H-S FUNCTION FOR PENDANT DROPS: S VS. $1/H$

$$S = \frac{d_s}{d_e}$$

S	0	0.001	0.002	0.003	0.004	0.005	0.006	0.007	0.008	0.009
0.70	0.797	0.795	0.792	0.790	0.787	0.785	0.782	0.780	0.777	0.775
0.71	0.772	0.770	0.767	0.765	0.762	0.759	0.757	0.754	0.752	0.749
0.72	0.747	0.744	0.742	0.739	0.737	0.735	0.732	0.730	0.727	0.725
0.73	0.722	0.720	0.718	0.715	0.713	0.711	0.709	0.706	0.704	0.702
0.74	0.699	0.697	0.695	0.692	0.690	0.688	0.685	0.683	0.681	0.679
0.75	0.676	0.674	0.672	0.670	0.668	0.665	0.663	0.661	0.659	0.657
0.76	0.655	0.652	0.650	0.648	0.646	0.644	0.642	0.640	0.637	0.635
0.77	0.633	0.631	0.629	0.627	0.625	0.623	0.621	0.619	0.617	0.615
0.78	0.613	0.611	0.609	0.607	0.605	0.603	0.601	0.599	0.597	0.595
0.79	0.593	0.591	0.589	0.587	0.585	0.583	0.581	0.579	0.577	0.575
0.80	0.573	0.571	0.570	0.568	0.566	0.564	0.562	0.561	0.559	0.557
0.81	0.555	0.553	0.551	0.550	0.548	0.546	0.544	0.542	0.540	0.539
0.82	0.537	0.535	0.533	0.532	0.530	0.528	0.526	0.524	0.523	0.521
0.83	0.519	0.518	0.516	0.515	0.513	0.511	0.509	0.507	0.506	0.504
0.84	0.502	0.501	0.499	0.498	0.496	0.494	0.493	0.491	0.490	0.488
0.85	0.486	0.485	0.483	0.482	0.480	0.479	0.477	0.476	0.474	0.473
0.86	0.471	0.470	0.468	0.467	0.465	0.464	0.462	0.461	0.459	0.458
0.87	0.457	0.455	0.454	0.452	0.451	0.450	0.448	0.447	0.446	0.444
0.88	0.443	0.441	0.440	0.439	0.437	0.436	0.434	0.433	0.431	0.430
0.89	0.429	0.428	0.426	0.425	0.423	0.422	0.420	0.419	0.418	0.417
0.90	0.415	0.414	0.413	0.411	0.410	0.409	0.408	0.406	0.405	0.404
0.91	0.403	0.401	0.400	0.399	0.398	0.396	0.395	0.394	0.393	0.391
0.92	0.390	0.389	0.388	0.386	0.385	0.384	0.383	0.382	0.381	0.379
0.93	0.378	0.377	0.376	0.375	0.373	0.372	0.371	0.370	0.369	0.369
0.94	0.366	0.365	0.364	0.363	0.362	0.361	0.359	0.358	0.357	0.356
0.95	0.355	0.354	0.353	0.352	0.351	0.350	0.349	0.348	0.346	0.345
0.96	0.344	0.343	0.342	0.341	0.340	0.339	0.338	0.337	0.335	0.334
0.97	0.333	0.332	0.331	0.330	0.329	0.328	0.327	0.326	0.325	0.324
0.98	0.323	0.322	0.321	0.320	0.319	0.318	0.317	0.316	0.315	0.314
0.99	0.313	0.312	0.311	0.310	0.309	0.308	0.307	0.306	0.305	0.304
1.00	0.303									

Accuracy: ±0.2%

of the measured surface tension extending over a period of several hours. It may be noted that the measured surface tension varied no more than ±0.5 dyne/cm., which is within the range of the accuracy of this method. Other tests on distilled water in uncontaminated air yielded similar results. Opening and closing the box (as would be necessary to introduce odorous material in the chamber) resulted in no significant break in the surface tension curve.

After it was established that the surface tension of distilled water is relatively stable, solid materials known to have strong odors were introduced into the box and the surface tension measurements continued. Figure 7 shows the change of surface tension resulting in one case after the introduction of cloves into the box and in another case after the introduction of gum camphor into the box. Curves *a* and *b* represent two independent runs with cloves as the contaminant, while curves *c* and *d* represent similar runs with camphor as the contaminant. Figure 8 shows the results of similar runs when three other materials—rubber, iodoform, and moth-balls—were used. It is of interest to note that the several materials produced different effects on the surface tension of the distilled water. Not only

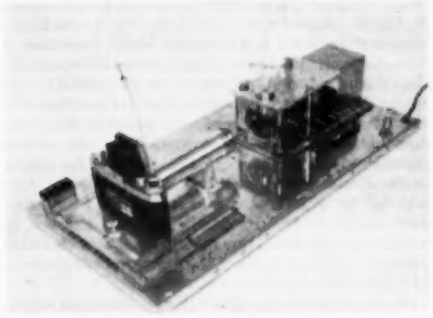


Fig. 2.—Photograph of instrument for measuring change of surface tension.

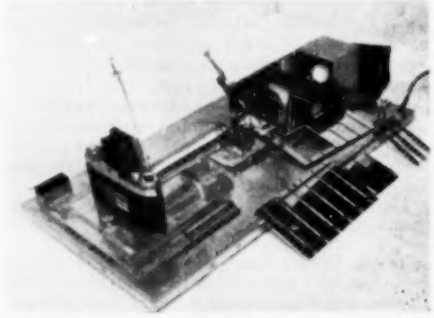


Fig. 3.—Odor chamber disassembled, ready for cleaning.

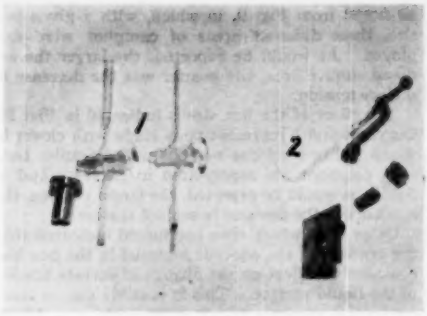
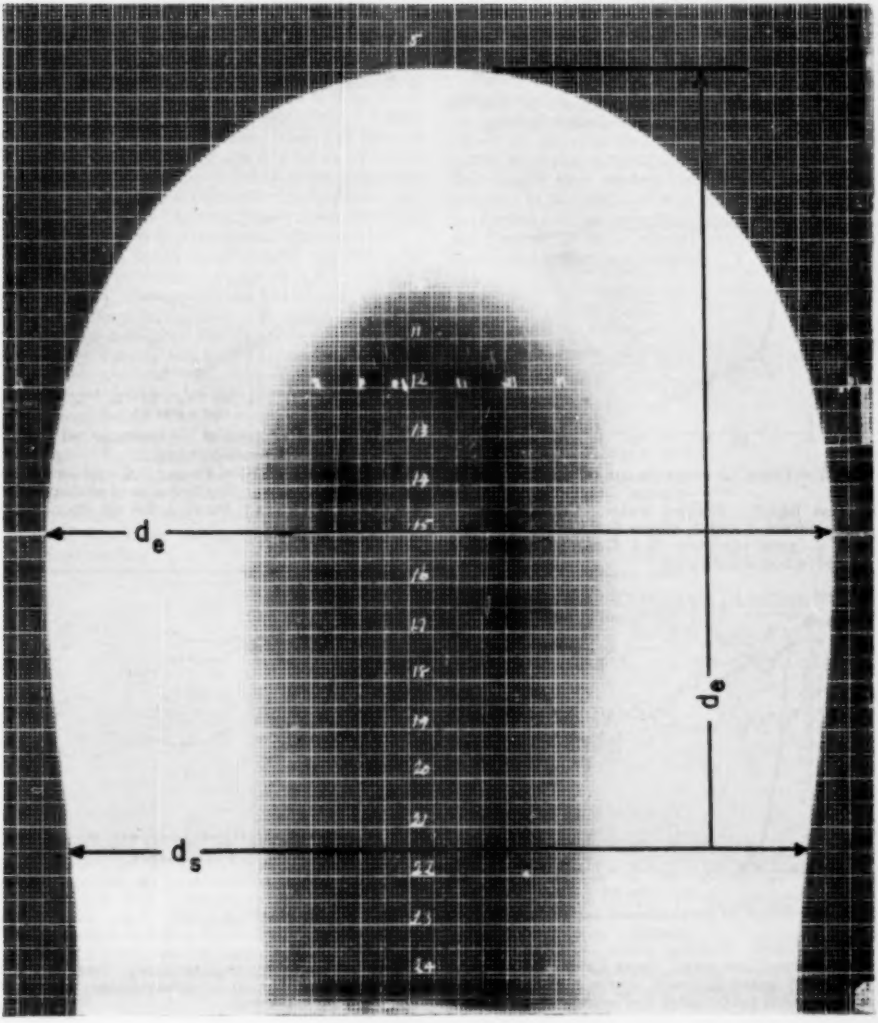


Fig. 4.—Droppers used in odor chamber.

does the surface tension change appreciably when the odorous material is introduced into the box, but certain periodic, though random, fluctuations in surface tension are observable. It is rather reasonable to expect that there might be a uniform change in surface tension due to these contaminants, but it is rather difficult to explain these random fluctuations. These fluctuations, in fact, were very sudden and apparently represented quite violent changes in the

Fig. 5 (*Below*).—Typical drop image with significant dimensions indicated.

d_e = 15.01 Units
d_s = 14.43 Units
S = 0.956
d_e = 0.415 Cm.
y = 60.10 dynes/cm.



surface condition of the drop. In many cases visual observation disclosed sudden mechanical movements of the drop definitely not associated with vibrations of the equipment.

As would be expected, the exposed area of the sample of odorous material produced a considerable effect on the change of surface tension. This may be noted from Fig. 9, in which, with a given box size, three different areas of camphor were employed. As would be expected, the larger the exposed surface area, the greater was the decrease in surface tension.

The effect of the box size is indicated in Fig. 10. Curves *a* and *b* represent tests made with cloves in which the box volume was changed. Similar tests with camphor are represented in curves *c* and *d*. Again, as would be expected, the larger the box, the smaller was the decrease in surface tension.

Other tests which were conducted indicated that the position of the odorous material in the box had considerable effect on the change of surface tension of the liquid surface. This is possibly due to small convection currents which were, without a doubt, present within the box.

In order to study the effect of contaminants on the surface tension of other liquids, the distilled water of the previous experiments was replaced by mineral oil. The curves of Fig. 11 represent almost no change of surface tension in mineral oil while there was present in the box a small container of water.

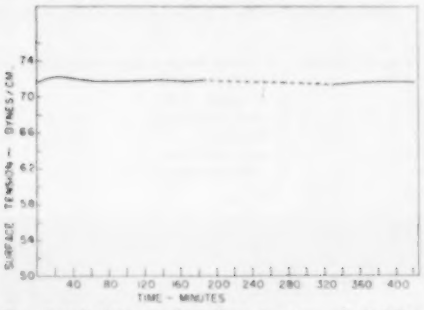


Fig. 6.—Variation of surface tension of distilled water. No contamination in odor chamber.

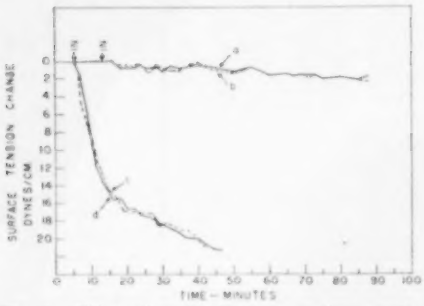


Fig. 7.—Effect of contaminant on surface tension change.

Test liquid: distilled water. Contaminant in box: curves *a* and *b*, cloves (1.7 Gm.); curves *c* and *d*, gum camphor (0.4 Gm.). Contaminant placed *in* box as indicated.

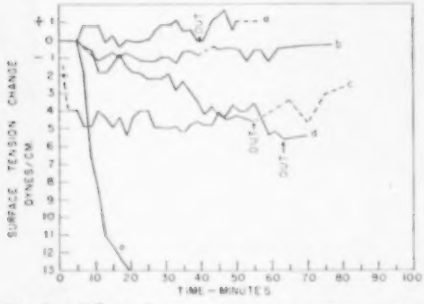


Fig. 8.—Effect of contaminant on surface tension change.

Test liquid: distilled water. Contaminant in box: curve *a*, iodoform; curve *b*, moth balls; curve *c*, rubber; curve *d*, cloves; curve *e*, gum camphor. Contaminant taken *out* of box as indicated.

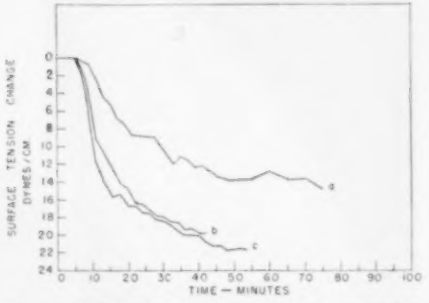


Fig. 9.—Effect of area of contaminant on surface tension change.

Test liquid: distilled water. Contaminant in box: gum camphor. Exposed area of contaminant: curve *a*, 1.3 sq. cm.; curve *b*, 2.4 sq. cm.; curve *c*, 6.0 sq. cm.

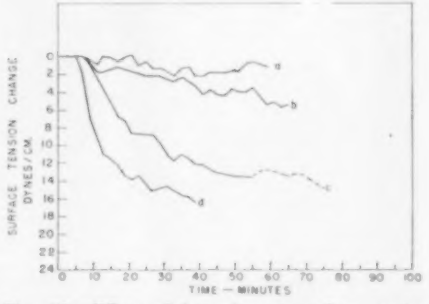


Fig. 10.—Effect of box size on surface tension change.

Test liquid: distilled water. Curve *a:* contaminant, cloves; box size 2 x 3 x 3 inches. Curve *b:* contaminant, cloves; box size 1 x 1.5 x 3 inches. Curve *c:* contaminant, camphor; box size 5 x 3 x 3 inches. Curve *d:* contaminant, camphor; box size 1 x 3 x 3 inches.

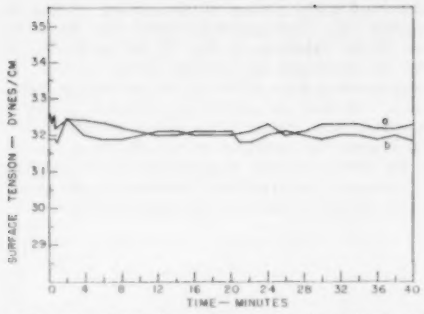


Fig. 11.—Variation of surface tension of mineral oil. Container of distilled water present in odor chamber. No other contamination. Curves *a* and *b*, two separate runs.

This water was added to the box because the odor-detecting equipment of all animals, as far as is known, operates in the presence of moist surfaces. It is obvious that the surface tension of mineral oil in a moist atmosphere is reasonably stable. Figure 12 shows the changes in surface tension of mineral oil when there is present in the box either rubber or iodoform. The magnitude of this surface tension is not greatly changed but some fluctuation in magnitude may be noted. Camphor and cloves produced little change in the surface tension of mineral oil.

Studies were made to determine the effects of odorants on the surface tension of a mercury surface. Figure 13 shows the surface tension of mercury for three different tests. It may be observed that immediately after the formation of the drop, the surface tension fell very rapidly, perhaps due to the absorption of water vapor on the mercury surface. By placing within the drop chamber a small container of distilled water, the surface tension of the mercury fell more rapidly and soon became relatively stable. As may be noted from this figure, in one test stability was reached at about 365 dynes/cm., and in two other tests at about 350 dynes/cm. This is substantially below the published figures for the surface tension of a mercury-to-air surface.

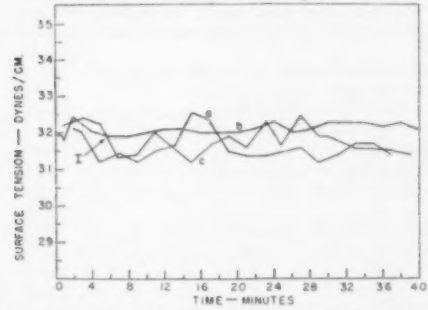


Fig. 12.—Effect of contaminant on surface tension change.

Test liquid: mineral oil. Contaminant: curve *a*, rubber; curve *b*, no contaminant; curve *c*, iodoform. Rubber sample placed in box before liquid drop was formed. Iodoform placed in box at point *I*.

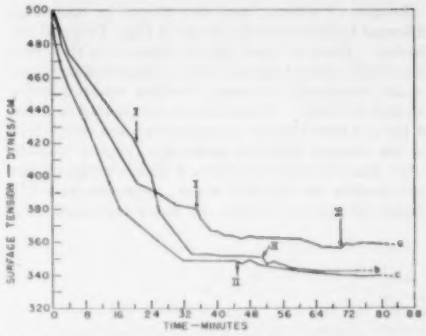


Fig. 13.—Variation of surface tension of mercury. Three separate tests.

Container of distilled water placed in odor chamber at *I*. Chamber re-opened and empty odor container placed in box at *II*.

Figure 14 shows the effect of iodoform in the chamber on a water-stabilized mercury drop. It may be noted that when the odorous material was added to the chamber, the surface tension fell rather promptly approximately 15 dynes/cm. and then varied in a random nature with time. The presence in the chamber of a quantity of camphor gave similar results as indicated in Fig. 15. Figures 16, 17, and 18 show similar tests for mothballs, cloves, and rubber, respectively.

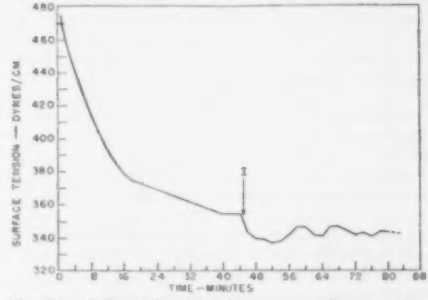


Fig. 14.—Effect of contaminant on surface tension. Test liquid: water-stabilized mercury. Contaminant: iodoform placed in chamber at *I*.

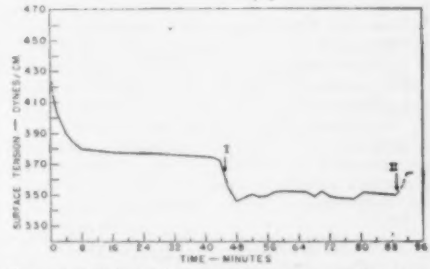


Fig. 15.—Effect of contamination on surface tension.

Test liquid: water-stabilized mercury. Contaminant: gum camphor placed in chamber at *I*.

Results of several tests are shown in somewhat different fashion by the curves of Figs. 19 to 23, inclusive. Each of these figures represents the effect of a single odorant on the surface tension of the three liquids employed; namely, distilled water, mineral oil, and mercury. These curves are plotted in terms of the per cent change in surface tension occasioned by the several different odorants. Figure 19 indicates that camphor produces a 20% change of surface tension on distilled water, approximately 6% change of surface tension on water-stabilized mercury, and a 3% change of the surface tension of mineral oil. Corresponding results are shown in Fig. 20 for iodoform, in Fig. 21 for mothballs, in Fig. 22 for cloves, and in Fig. 23 for rubber. A comparison of Figs. 19 and 22 is particularly interesting. It may be noted that camphor causes a relatively large change in the surface tension of distilled water as compared to the change produced in the surface tension of water-stabilized mercury. In contrast, cloves produce a relatively large change in the surface tension of the water-stabilized mer-

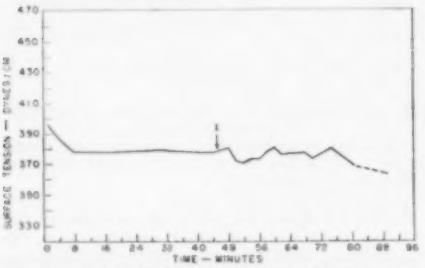


Fig. 16.—Effect of contamination on surface tension. Test liquid: water-stabilized mercury. Contaminant: moth ball placed in chamber at *I*.

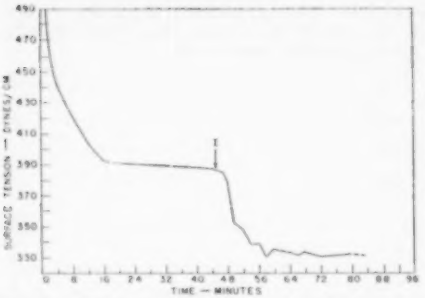


Fig. 17.—Effect of contamination on surface tension. Test liquid: water-stabilized mercury. Contaminant: cloves placed in chamber at *I*.

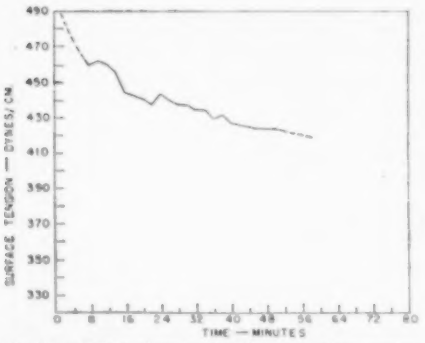


Fig. 18.—Effect of contamination on surface tension. Test liquid: water-stabilized mercury. Contaminant: rubber placed in chamber twenty-three hours before mercury drop was formed.

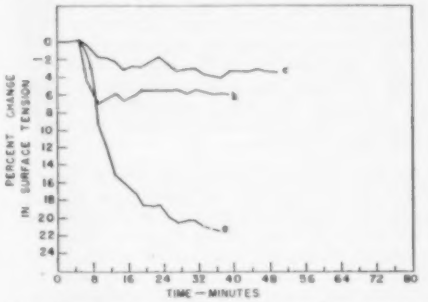


Fig. 19.—Per cent change in surface tension. Contaminant: gum camphor. Test liquids: curve *a*, distilled water; curve *b*, mercury; curve *c*, mineral oil.

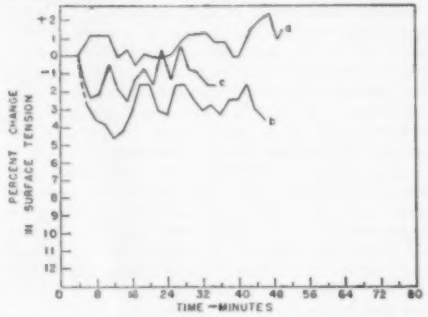


Fig. 20.—Per cent change in surface tension. Contaminant: iodoform. Test liquids: curve *a*, distilled water; curve *b*, mercury; curve *c*, mineral oil.

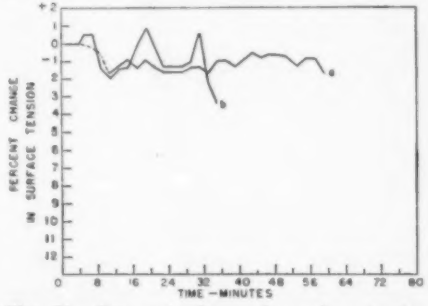


Fig. 21.—Per cent change in surface tension. Contaminant: moth balls. Test liquids: curve *a*, distilled water; curve *b*, mercury.

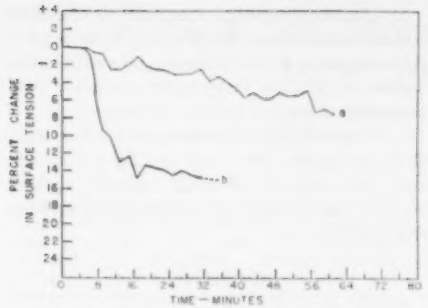


Fig. 22.—Per cent change in surface tension. Contaminant: cloves. Test liquids: curve *a*, distilled water; curve *b*, mercury.

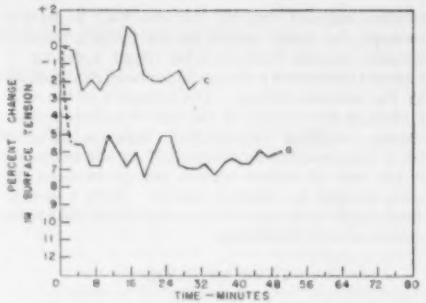


Fig. 23.—Per cent change in surface tension. Contaminant: rubber. Test liquids: curve *a*, distilled water; curve *c*, mineral oil.

cury as compared with the effect of cloves on distilled water. An analysis of these two curves indicates that by a comparison of the changes produced in the surface tension of the two drops, water and water-stabilized mercury, it would be possible to discriminate between the presence in the test chamber of camphor or cloves. Similar conclusions might be reached relative to the other odorous materials employed.

Correlation of Surface Tension Experiments with Olfaction.—It is of interest to note that some of the characteristics of the surface tension curves are similar to the known characteristics of olfaction. These similarities suggest that possibly the mechanism of olfaction is in some way associated with the changes in surface tension of some of the liquids of the nose due to the presence in the nose of molecules of odorous substances. Points of similarity which might be mentioned are as follows:

1. The decrease of the surface tension of a liquid as effected by odorous materials continues for several minutes and then becomes stable. This is somewhat in agreement with the character of the nose in that odors can be detected for a period of time, but with the onset of fatigue of the olfactory system, the odors are no longer detectable. This suggests that odors may possibly be detected by the nose during the period when surface tension is changing.

2. The rate of change of surface tension is dependent on the exposed odorous materials area which presumably determines the rate at which the molecules in the surface layer are forming on the drop. Similarly the nose detects the presence of a large quantity of odorous material as contrasted to small concentrations and more quickly becomes fatigued.

3. The surface tension curves show some variation in value even after the curves have reached a more or less stable reduced value. These low amplitude swings in the surface tension curves suggest the observed behavior of the nose which is able occasionally to detect an odor even when it has become fatigued to the odor over a considerable period of time.

4. As shown in certain tests, data of which are not presented herein, the removal of the odorous material from the drop chamber caused the surface tension to start rising immediately and to restore to practically normal value within a period of five or ten minutes. As is well known, the nose, after being fatigued to an odor, will regain its sensitivity to that odor after being supplied with fresh air for a few minutes.

5. No tests were run to examine the change in surface tension of the liquid drop in the presence of more than one odorant. It seems reasonable to believe that one material might be placed in the box and cause relatively small surface tension change, but that the addition of a second odorant might cause a further reduction in surface tension. If so, this behavior would be in agreement with that of the nose in which fatigue may develop with reference to one odor, yet leave the nose able to detect readily another odor which might be presented.

6. On the basis of a camphor molecule having a diameter of 2.8×10^{-8} cm. forming a monomolecular layer on a spherical water drop of diameter 0.4 cm., approximately 2×10^{15} molecules would be required. As indicated by studies made by Passy, the minimum number of molecules of camphor which must be drawn into the nose to produce the sensation of odor is approximately 4×10^{14} molecules. That these two figures should be of the same order of magnitude, is of more than passing interest.

7. The effect of contamination on the surface tension of a liquid has been shown to be associated with the architectural characteristics of the molecule of the contaminant (13, 18–20). While this particular phase of the work has not been studied in the present experiments, other investigators have shown this to be the case where contamination was added directly to the liquid. Numerous investigators have shown somewhat similar relations existing between odors and molecular structures. For instance, structurally similar isomers usually have similar odors. As the difference in molecular structure or architectural style of isomers increases, so do odor differences. Some stereoisomers have different odors.

The surface tension experiments indicate a possible mechanism of olfaction. It is possible that in the nose there is disposed over certain areas small patches of different liquids, and perhaps over all of these is a coating of lipoid fluid. Molecules of

odorous material entering the nose may be passed through the lipoid liquid to the surface tension-sensitive liquids there to bring about a change in surface tension and a change in pressure recognizable by the nervous system. The intensity of the odor is perhaps determined by the rate of surface tension change resulting. Distinction between different odors may possibly be determined by the difference in the rate of surface tension change between the areas covered by different liquids. Such a mechanism would be in agreement with many of the known characteristics of olfaction.

CONCLUSIONS

1. The surface tension of several different liquids has been shown to change due to airborne contamination from nearby solid odorous materials. The amount of the surface tension change has been readily measured by the Pendant Drop Method. The degree of contamination used in the experiments has been that which would be classified as a strong odor.

2. The effect on the surface tension of contamination of the air by different odorants is characteristic of the odorous materials and is quite different on the three liquid surfaces considered in the experiments.

3. The change in the surface tension of the liquid due to contaminated atmosphere is influenced by the degree of contamination.

4. There have been presented certain similarities between the behavior of liquid surfaces in the presence of contaminated atmosphere and olfaction.

5. An improvement in the method of surface tension measurement which would make possible the measurement by electrical means with amplification might show many more interesting characteristics of surface tension phenomena.

6. These studies extended with greater sensitivity to liquids other than distilled water, mercury, and mineral oil might indicate methods by which many different contaminations could be differentiated.

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WHO MAKES IT?

The National Registry of Rare Chemicals, Armour Research Foundation, 33rd, Federal and Dearborn Streets, Chicago, Ill., seeks information on sources of supply for the following chemicals:

Strontium tartrate tetrahydrate
2-Carboxy-5-*n*-butyl pyridine
1,5-Dimethyl imidazole
1,5-Dimethyl iminazole
d- or *l*-Ethyl lactate
1-Methyl-5-cyanomethyl imidazole
Tetramethylhydrazine
3-Hydroxy-N-methylmorphinan
1-(*p*-Methoxyphenyl)-1,2-epoxypropane
N,N-Dimethylthioacetamide
4-Amino-2-carboxy-1-N-phenyl-4-amino-4′-oxy-1,1′-diphenylmethane
2-Amino-5-nitrothiazole
2-*n*-Butyl hydroquinone
Carvacrol, pure
Emodin
Fraserin
Phytolaccinum
Protoanemonin
Ptelein

A Study of Monoalkylcarbamates as Local Anesthetics*

By D. A. SCHLICHTING,† G. E. CWALINA, and G. L. JENKINS‡

A series of 21 new monoalkylcarbamic acid esters of some tertiary-amino alcohols has been prepared. Two new ureas have been isolated and identified. Single pharmacological tests have been made with each compound using three methods: the rabbit cornea, the guinea pig wheal, and the Rider frog. The results indicate that some of the compounds are local anesthetics and that further pharmacological study of the series is warranted.

A NUMBER of studies have been made of the local anesthetic activity of arylcarbamic acid esters of aminoalcohols. Only three references to analogous monoalkylcarbamic acid esters have been found in the literature (1–3). Of these, 2-diethylaminoethyl 2-propylcarbamate (2) was tested as a local anesthetic; however, no specific statement was made concerning its activity. In the present investigation we have undertaken the synthesis of a series of these compounds, the majority of which are of the general type, $RNHCO_2C_nH_{2n}NR_2$. The work accomplished extends this series to the higher alkyl-, branched alkyl-, and unsaturated alkylcarbamic acid esters of some of the more important tertiary-amino alcohols. The general reactions involved in their synthesis are written below in which R = alkyl and R′ = dialkylaminoalkyl except for 3-piperidino-1,2-propanediol monononylcarbamate in which R′ is the piperidinoalkyl.

$$RCO_2H \xrightarrow{SOCl_2} RCOCl \xrightarrow{NaN_3} RCON_3 \xrightarrow{heat} RNCO$$

$$RNCO + HOR' \longrightarrow RNHCO_2R'$$

EXPERIMENTAL

The methods used in the synthesis of 2-diethylaminoethyl heptylcarbamate exemplify the general methods used throughout the work.

Preparation of Octanoyl Chloride.—The acid chloride is prepared from 29.5 Gm. (0.25 mole) of thionyl chloride and 33.0 Gm. (0.23 mole) of octanoic acid without the use of a solvent. A yield of 32.3 Gm. (0.20 mole) of octanoyl chloride, b. p. 90–95°/15 mm., is obtained. This represents a yield of 87% of the theoretical based on the acid used.

Preparation of Heptyl Isocyanate.—A mixture of 17.0 Gm. (0.26 mole) of activated sodium azide and 50 cc. of toluene, previously dried over sodium and distilled, is reacted with 32.3 Gm. (0.20 mole) of octanoyl chloride as in the Curtius reaction (4). Slight heating of the resultant mixture initiates a reaction which lasts an hour. The mixture is then heated for an additional ninety minutes, after which it is cooled and filtered and the toluene removed *in vacuo*. Distillation gives 17.5 Gm. (0.12 mole) of a colorless, limpid, sweet-smelling, lacrymatory liquid which boils at 83.5°/15 mm. The yield based on octanoyl chloride is 60%.

Preparation of 2-Diethylaminoethyl Heptylcarbamate.—To 14.2 Gm. (0.10 mole) of heptyl isocyanate in 25 cc. of dry toluene is added 14.0 Gm. (0.12 mole) of 2-diethylaminoethanol. Dry hydrogen chloride gas is later passed into half of the toluene solution for thirty minutes and the product is allowed to stand overnight. Additional HCl gives no further precipitation. The precipitate is collected giving 2.1 Gm. of a product which is recrystallized from chloroform twice and from dry acetone four times, m. p. 127.5 to 130.5°. The remaining toluene solution of the free base is fractionated and 6.4 Gm. of a product, b. p. 156–157°/1 mm., is obtained. The yield is 32% of the theoretical based on the isocyanate.

The dihydrogencitrate is prepared by adding a saturated solution of citric acid to the free base dissolved in dry ether until precipitation is completed. The citric acid solution is prepared by dissolving the acid in a mixture of 10% anhydrous methanol and 90% anhydrous ether. The above precipitate is washed several times with dry ether or petroleum ether and then recrystallized several times from a mixture of ethyl acetate and anhydrous methanol (20:1). The product is a white crystalline material, m. p. 100.5 to 101.5°.

Anal.—Calcd. for $C_{20}H_{38}O_9N_2$: N, 6.22. Found: N, 6.20; 5.81.

DISCUSSION

In most syntheses it was necessary to reflux the acid azide for three hours in order to complete the decomposition to isocyanate. Attempts to prepare isocyanates in a benzene solution were unsuccessful. Repetition of one such unsuccessful attempt using toluene instead of benzene was successful. The additional heat thus afforded is apparently desirable for efficient formation of the acid azide as well as for its subsequent decomposition.

* Received May 5, 1950, from the Research Laboratories, Purdue University, School of Pharmacy, Lafayette, Ind.
Presented to the Scientific Section A. Ph. A., Atlantic City meeting, May, 1950.

† American Foundation for Pharmaceutical Education Fellow, 1947–1949. Present address: The Wm. S. Merrell Co., Cincinnati, Ohio.
‡ The authors are indebted to Dr. L. D. Edwards for his suggestions concerning the pharmacological work and to the Wm. S. Merrell Co. and Rohm and Haas, Inc., for supplying some of the materials.

TABLE I.—SUMMARY OF EXPERIMENTAL DATA OBTAINED

Compound Prepared	B. P. and/or M. P.	HCl Salt M. P.	Citrate M. P.	Analysis for Nitrogen[a] Calcd.	Found	Yield %
2-Diethylaminoethyl butylcarbamate	124.5–126.5°/ 2 mm.	91.5–94.5	...	11.08	11.31[b]	74
3-Diethylaminopropyl butylcarbamate		108.5–113.0	55.0–55.0	10.51	10.26	
2-Diethylaminoethyl pentylcarbamate	157.5–159.5°/ 2.3 mm.	...	105.5–106.5	12.16	11.91[b]	68
3-Diethylaminopropyl pentylcarbamate	166.0–167.5°/ 2 mm.	...	65.5–67.5	6.42	6.21[c]	80
2-Diethylaminoethyl hexylcarbamate	136.5/2 mm.	...	101.0–102.5	11.47	11.28[b]	51
3-Diethylaminopropyl hexylcarbamate	150.5–152.5°/ 2 mm.	...	...	10.85	10.75[b]	62
2-Diethylaminoethyl heptylcarbamate	156.0–157.0°/ 1 mm.	127.5–130.5	100.0–101.5	6.22	6.01[c]	32
3-Diethylaminopropyl heptylcarbamate	m. p. 90.0–91.5	...	77.5–80.5	10.29	10.48[b]	93
2-Diethylaminoethyl octylcarbamate	...	...	64.0–96.0	6.03	6.12[c]	90
3-Diethylaminopropyl octylcarbamate	...	...	80.5–82.5	5.85	5.80[c]	92
2-Dimethylaminoethyl octylcarbamate		95.0–97.0	...	9.98	10.06[b]	92
2-Diethylaminoethyl nonylcarbamate	m. p. 88.5–93.5	97.5–99.5	104.5–106.0	8.68	8.57[b]	78
3-Diethylaminopropyl nonylcarbamate	m. p. 90.5–97.5	...	76.0–77.5	9.32	9.21[b]	80
3-Piperidino-1,2-propanediol monononylcarbamate		97.0–102.5	...	7.69	7.68[b]	92
2-Diethylaminoethyl 9-decenylcarbamate	...	127.5–130.5	...	8.67	8.15[b]	7
2-Diethylaminoethyl 3-pentylcarbamate	122.5–124.5°/ 2 mm.	...	80.5–82.5	12.16	12.09[b]	62
3-Diethylaminopropyl 3-pentylcarbamate	140.0–144.0°/ 4 mm.	...	97.0–99.5	11.46	11.14[b]	57
2-Diethylaminopropyl 3-pentylcarbamate	113.5–116.5°/ 2 mm.	...	...	11.46	11.20[b]	57
2-Diethylaminoethyl 2,4,4-trimethylpentylcarbamate	146.5–147.5°/ 10 mm.	108.0–109.5	...	9.67	8.88[b]	51
3-Diethylaminopropyl 2,4,4-trimethylpentylcarbamate	151.5–152.5°/ 13 mm.	...	82.5–86.5	5.85	5.85[d]	70
2-Dimethylaminoethyl 2,4,4-trimethylpentylcarbamate	126.5–128.5°/ 8 mm.	124.5–126.5	...	9.98	9.97[b]	53

[a] All analyses were made at least in duplicate and the values reported to two decimal places are given.
[b] Analyzed as the free base.
[c] Analyzed as the hydrochloride.
[d] Analyzed as the dihydrogen citrate.

Isocyanates were isolated in the crude form several times but distillation did not give analytically pure products. It is suggested that the isocyanates be used directly for the next step, without attempting isolation. Their reactions with aminoalcohols were carried out at room temperatures for from twenty-four to forty-eight hours in most cases.

Carbamates prepared in commercial anhydrous ether solution more often contain appreciable amounts of symmetrical ureas as impurities than do those prepared in anhydrous toluene. Apparently sufficient moisture is present in the former to cause the reaction $2RNCO + H_2O \rightarrow RNHC(O)NHR + CO_2$ to occur. Nonhygroscopic solvents, properly dried, will eliminate this side reaction to a great extent. N,N'-Dihexylurea and N,N'-dinonylurea were isolated and identified in two instances where ether was used as a solvent.

Product	M. P.	Analysis for N Calcd.	Found
N,N'-dihexylurea	74.5–75.5°	12.27	12.29
N,N'-dinonylurea	96.0–97.5°	8.96	8.75

The lower molecular weight carbamates can be purified by distillation at reduced pressures. Some of the higher molecular weight carbamates can be crystallized as the free bases. Although some hydrochloric acid salts were successfully prepared, the citric acid salts are more readily ob-

tained. The hydrochlorides were best prepared by adding a cold, dry ethereal solution of hydrochloric acid to a cold, dry ethereal solution of the free base. The results obtained are summarized in Table I.

Preliminary pharmacological testing was carried out using the rabbit cornea method (5), the guinea pig wheal method (6), and the Rider frog method (7). Single tests were made on each new carbamate using all three methods in an attempt to determine whether further study of the compounds as local anesthetics is warranted. The results indicate that some of the compounds are local anesthetics and that further pharmacological investigation of the series is warranted.

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Studies on *Mahonia* Genus. II. Botany and Chemistry of *M. acanthifolia* Don*

By R. CHATTERJEE and M. P. GUHA†

***Mahonia acanthifolia* Don is usually confused with *M. nepalensis* D. C. It has been shown by the isolation of the alkaloids of *M. acanthifolia* that this plant may be differentiated from *M. nepalensis*.**

THE species *M. acanthifolia* (family *Berberidaceae*) was described first in 1830 by Don (1) who recognized this as a species distinct from *M. nepalensis* of De Candolle (2). In 1855, Hooker and Thomson (3), however, described the present *M. nepalensis* as *Berberis nepalensis* Spreng and changed *M. acanthifolia* to *B. nepalensis*. Since then all the later workers followed this opinion until 1902, when Fedde (4) recommended that *Mahonia* should be treated separately from *Berberis*. The former genus was distinguished from the latter by its imparipinnate leaves, the terminal inflorescence, and the absence of spines (5). Since then only one species of *Mahonia*, e.g., *M. nepalensis*, was known to be a native of India, and this species was supposed to be distributed over the whole range of the Himalayas, Nepal, and Assam. According to Takeda (6), true *M. nepalensis* had never been found outside Nepal and true specimens were those collected from Nepal by Buchanon in 1802 and by Wallich in 1821. *Mahonia acanthifolia* described by Don (1), which was formerly thought to be synonymous with *M. nepalensis* D. C., was found to be most widely distributed from Nepal, Darjeeling to Assam, and Kumaon. The present authors, however, collected specimens of *M. nepalensis* from Darjeeling where it grew side by side with *M. acanthifolia*; the former species was of stunted growth and did not usually flower in Darjeeling. A field observation of the living plants of both the species, which are not easily distinguished without examination of flowers, showed that the midribs of the young leaves and young leaflets of *M. nepalensis* are coppery red in color which lasted for a year or so and disappeared as the leaflets and leaves matured. Those of *M. acanthifolia*, however, were greenish white.

Mahonia acanthifolia could be distinguished from *M. nepalensis*, by an examination of their flowers. In the absence of flowers, it appeared difficult to identify the mature plants from the leaflets. Table I shows the points of difference of these two plants.

Both the species show great similarities in their anatomical structures and in the distribution of the alkaloids in the different cells. The fibrous and main roots showed that the outer cells of cortical tissues contained a fair concentration of the alkaloids, whereas the central cells were comparatively free. Many isolated cells of the cortex, cells on the inner side of the phellogen, and cell walls near the pith showed a larger accumulation of the alkaloids.

* Received December 10, 1949, from the Departments of Chemistry, University College of Science and Technology, and Presidency College, Calcutta, India.
† The authors are indebted to Dr. S. K. Mukerjee, Herbarium Curator, Royal Botanic Garden, Sibpur, Calcutta, for critical suggestions on botanical matter and to Dr. N. K. Sen, Head of the Department of Chemistry, Presidency College, for providing them with laboratory facilities. R. Chatterjee acknowledges with thanks an ICI Research Fellowship of the National Institute of Sciences of India, during the progress of this work.

TABLE I.—THE POINTS OF DIFFERENCE BETWEEN *M. nepalensis* D. C. AND *M. acanthifolia* DON

	M. nepalensis	*M. acanthifolia*
Flowers	Anthers equal to filaments	Anthers much shorter than filaments
Berries	Style insignificant	Style 1 mm., conspicuous, coronate
Leaflets	Usually 7 pairs, 5–10 teeth in lower and 4–7 in upper leaflets	About 11 pairs, 3–6 teeth in lower and 2–5 in upper leaflets

In the stems, the alkaloids were deposited in the medullary rays, in the cells of the xylem, and the hard basts of the phloem. The cells of the pith were also found to contain the alkaloids.

The midribs of the leaves and leaflets contained the alkaloids in sclerenchymatous tissues around the vascular bundles. The cells of the mesophyll were, however, free from alkaloids.

Apart from the subtle morphological differences of these two species of *Mahonia*, the difference in quantity and nature of the alkaloids obtained from these two species growing in the same locality, had strongly supported the view of Don (1) that *M. acanthifolia* is quite distinct from *M. nepalensis*. Chatterjee could isolate the alkaloids, umbellatine and neprotine from the latter (7), whereas five alkaloids, oxyacanthine, berberine, neprotine, palmatine, and jatrorrhizine had been recorded from the former species. *Mahonia acanthifolia* also appeared to be chemically different from *M. Griffithii* Takeda (8) for berbamine could not be identified in *M. acanthifolia* and jatrorrhizine was not obtained from *M. Griffithii*.

EXPERIMENTAL

The plant material was obtained from Darjeeling by courtesy of B. N. Ghosh, Townend, Darjeeling, and was identified in consultation with Dr. S. K. Mukerjee, Herbarium Curator, Royal Botanic Garden, Sibpur, Calcutta.

Dried, finely powdered root of *M. acanthifolia* (1 kg.) was extracted exhaustively with warm ethanol (95%). The solvent was removed under reduced pressure and the sticky brown mass residue was treated with iced water (500 cc.) and filtered three times to remove resinous matter. The clear dark brown liquid was cooled to 0° and made alkaline with ammonia (25%), when a sticky amorphous solid separated and the mixture was extracted five times with ether.

Treatment of the Ether Layer

The yellow-colored ether solution was washed twice with water, and on removal of ether, a sticky resinous residue (1 Gm.) was left behind. This was extracted in a Soxhlet apparatus with dry ether (50 cc.) until most of it dissolved. The solution was filtered and kept for three days in a refrigerator until a very small amount of colorless crystals had separated. These were treated with a small quantity of dilute hydrochloric acid (5%), filtered from insoluble matter and kept in a refrigerator until crystals of a hydrochloride separated out.

The Crystals, Identification of Oxyacanthine.—The pale yellow crude hydrochloride of the base was dissolved in warm water and was treated with ammonia to precipitate an amorphous base. This was taken up in ether, after which the ethereal solution, on long standing in the refrigerator, yielded a very small quantity of colorless crystals which on recrystallization from methanol melted at 216° *in vacuo* and did not depress the melting point of an authentic sample of oxyacanthine, m. p. 216° *in vacuo* and showed the same color reactions as those of oxyacanthine.

The Mother Liquor.—The mother liquor could not be worked up successfully.

Treatment of the Aqueous Layer

The clear brown aqueous solution (600 cc.) was treated with hydrochloric acid to give a 1% acid solution. This solution was allowed to stand in the refrigerator overnight until a mixture of yellow and red hydrochlorides gradually crystallized. The crystals were then filtered off.

The Crystals, Separation of Berberine and Neprotine Hydrochlorides.—The mixture of the hydrochlorides (4 Gm.) was fractionally crystallized from ethanol to obtain crystals of yellow hydrochloride (3.8 Gm.) and of red hydrochloride (0.05 Gm.).

The yellow hydrochloride was further purified by several crystallizations from dilute alcohol. It did not show any definite melting point. The purified hydrochloride (0.3 Gm.) was dissolved in warm dilute hydrochloric acid and was treated with platinic chloride (0.15 Gm.) to precipitate the yellow chloroplatinate which was well washed with water until free from hydrochloric acid.

Anal.—Calcd. for $(C_{20}H_{19}O_4N)_2H_2PtCl_6$: Pt, 18.02; found, 18.03.

This indicated that the yellow hydrochloride might be berberinium chloride, which was further confirmed by the preparation and identification of the acetone compound of the base, as berberine acetone. The yellow hydrochloride (0.2 Gm.) was dissolved in water (20 cc.) and was mixed with 10% aqueous sodium hydroxide solution (4 cc.), warmed to 50° and mixed with acetone (10 cc.) and allowed to cool to the room temperature, when lemon-yellow compound of the acetone compound (0.12 Gm.) gradually separated. The crystals melted at 167–168° with decomposition, and did not depress the melting point of berberine acetone, m. p. 167–168° dec.

It was found that free berberine as liberated by the action of sodium carbonate did not furnish the acetone compound when crystallized from acetone. It seemed, therefore, that the presence of a caustic alkali is essential for the formation of the above compound.

The yellow hydrochloride (4 Gm.) in water (150 cc.) was reduced by zinc dust and acetic acid until the solution became faintly yellow. This was cooled and treated with ammonia until the precipitated zinc hydroxide redissolved. The ammoni-

acal solution was then repeatedly extracted with ether. The ether solution, on concentration, deposited crystals of a colorless compound (3 Gm.) identified to be tetrahydroanhydroberberine. The colorless compound was further crystallized from methanol, m. p. 172–173°. It did not lower the melting point of an authentic sample of tetrahydroanhydroberberine, m. p. 175°.

The crude red hydrochloride was purified by repeated crystallization from dilute ethanol and obtained as coppery red, beautiful elongated prisms on slow crystallization. The crystals charred without melting.

An ethanolic solution of picric acid (1 mole) was added to a warm ethanolic solution of the hydrochloride (1 mole) and on cooling, shiny orange-colored crystalline needles of the picrate, m. p. 208–210°, dec., were obtained. These did not lower the melting point of neprotine picrate, m. p. 208–210°, dec., which had been isolated similarly from *M. nepalensis* (8).

Berberine and neprotine hydrochlorides were subsequently separated more successfully by the following method based on the observation that berberine forms an insoluble acetone compound in alkaline medium, whereas neprotine remains in solution unaffected (8).

A solution of a mixture of the berberine and neprotine hydrochlorides (1.18 Gm.) in water (80 cc.) was warmed to 50° and was made alkaline with 10% sodium hydroxide solution (16 cc.). This was treated with acetone (40 cc.) and the mixture allowed to cool, when glistening yellow crystals of berberine acetone (0.235 Gm.) separated and these were removed by filtration. The filtrate was acidified by adding the least quantity of concentrated hydrochloric acid dropwise, and the solution was allowed to evaporate slowly at the room temperature, when shiny red crystals of neprotine hydrochloride (0.56 Gm.) were deposited after a week.

Treatment of the Filtrate.—The filtrate (600 cc.), after separation of berberine and neprotine hydrochlorides, was treated with sodium acetate solution, then a solution of potassium iodide, which precipitated the remaining bases as iodide (5.6 Gm.) which was filtered and the filtrate rejected.

The precipitated yellow-colored iodide (5.6 Gm.) associated with sticky matter was suspended in water (250 cc.) and was digested with freshly precipitated silver chloride (8 Gm.) on a sand bath for twenty-four hours. By double decomposition the hydrochloride of the bases was formed in the solution, while silver iodide remained in suspension. The mixture was filtered hot and the dark brown aqueous extract was immediately reduced by zinc dust with a mixture of glacial acetic acid (40 cc.) and concentrated hydrochloric acid (25 cc.). The mixture was warmed on a water bath until its color became very pale yellow. The reduced solution was filtered, cooled in ice, and was treated with ammonia until the precipitated zinc hydroxide was obtained. This was collected by filtration. The filtrate was extracted several times with ether. The pale yellow base was then dissolved by vigorous shaking with ether, and both the ether layers were mixed together; then washed with water, followed by washing with 5% sodium hydroxide solution (30 cc.). The ethereal layer was then washed with water until free from alkali.

Treatment of the Ether Solution, Identification of Palmatine

The residue (0.48 Gm.) obtained on removal of ether was crystallized from methanol to yield colorless needles of tetrahydropalmatine, m. p. 142–143°, and producing no depression in melting point when mixed with an authentic sample of tetrahydropalmatine, m. p. 144°.

It was found that the impure product rapidly turned yellow in contact with air, so the crystallization was carried out in an inert atmosphere. It was, however, noticed that after three crystallizations from methanol, the product did not become yellow, even in contact with air for a long time.

Oxidation by Iodine in Ethanol. The crystals (0.1 Gm.), believed to be of tetrahydropalmatine, were treated with a mixture of iodine (0.2 Gm.) and ethanol (4 cc.) in a sealed tube according to Spath, *et al.* (9) at 100° for three hours. The residue obtained on removal of the solvent was treated with a solution of sodium bisulfite to free it from iodine. This was then crystallized from water and from ethanol. On rapid heating at the ordinary pressure, the golden yellow crystals (0.15 Gm.) of palmatine iodide obtained turned brown at 210°, black at 230°, and melted with frothing and foaming at 260–262°. Literature records the melting point of palmatine iodide as 258–259° with decomposition *in vacuo*. At the ordinary pressure, however, an authentic sample of palmatine iodide behaved similarly to the above compound, and melted at 260–262°, dec.

Tetrahydropalmatine Hydrochloride.—Tetrahydropalmatine hydrochloride is not adequately described in literature. The free base tetrahydropalmatine is practically insoluble in water; the hydrochloride was, therefore, prepared by heating the isolated tetrahydro-compound with a small quantity of water. To this a few drops of dilute hydrochloric acid were then added, whereupon the hydrochloride dissolved immediately in hot water, and slowly crystallized out upon cooling. The recrystallized product sintered at 210° and melted at 216–218°, dec.

Treatment of the Alkaline Solution, Identification of Jatrorrhizine

The reduced product in alkaline solution (30 cc.) was dark brown in color, showing the presence of a phenolic base. As the color of the solution darkened on standing, the solution was cooled immediately in ice and was acidified carefully with concentrated hydrochloric acid. The hydrochloride (1.12 Gm.) precipitated as a pale yellow solid.

The Hydrochloride.—The hydrochloride, m. p. 240–241°, dec., was obtained as colorless crystals from methanol. A portion of the hydrochloride was dissolved in water and then made alkaline with ammonia. The crude base which did not keep well in air melted at 205–206°, dec. Literature (10) records the melting point of pure tetrahydrojatrorrhizine as 212–214°.

Oxidation of the Reduced Base by Iodine Tincture.—The crude reduced base was dissolved in methanol. It was treated with iodine in potassium iodide solution and methanol and heated on a water bath for one minute, and then filtered. The filtrate was acidified with dilute hydrochloric acid and as

TABLE II.—THE AMOUNT OF THE ALKALOIDS IN DIFFERENT PARTS OF *M. acanthifolia* DOX

Part Examined	Collected During	Yields of Crude Fractions in Gm./Kg. and Their Alkaloid Content in Gm./Kg. of the Air Dried Plant Material					
		Portion Soluble in Ether		Portion Soluble in Water			
		(I) Sticky Matter	(II) Alkaloid from (I)	(III) R. HCl	(IV) Alkaloids from (III)	(V) R. III	(VI) Alkaloids from (V)
Old plant							
1. Root	June	5.0	In traces[a]	5.78	−5.0[b]	9.06	−0.72[c]
					In traces[d]		−1.5[e]
	Dec.	2.5	In traces[a]	6.0	−5.3[b]	8.4	−0.71[c]
					−0.075[d]		−1.4[e]
2. Stem bark	June	1.1		3.2	−3.0[b]	5.5	−0.48[c]
					In traces[d]		−1.12[e]
3. Stem	Oct.	2.5	In traces[a]	3.0	−2.5[b]	7.5	−0.63[c]
					−0.03[d]		−1.25[e]
Young plant							
1. Root	Dec.	1.0	In traces[a]	1.0	−3.8[b]	5.6	−0.48[c]
					−0.05[d]		−1.12[e]
2. Root and stem bark	June	0.9		2.5	−2.2[b]	5.2	−0.40[c]
					In traces[d]		−1.0[e]
3. Stem bark	June	0.6		1.2	−1.0[b]	4.7	−0.35[c]
					In traces[d]		−0.92[e]
	Dec.	0.5		2.0	−1.8[b]	4.5	−0.35[c]
					In traces[d]		−0.90[e]

[a] Oxyacanthine. [b] Berberine, HCl. [c] Neoprotine, HCl. [d] Tetrahydropalmatine. [e] Tetrahydroisatisoridine.

the solution cooled, crystals of an iodide, jatrorrhizine iodide, separated as highly crystalline orange needles, which softened at 207° and melted at 210°. The crystals were washed with sodium bisulfite solution and crystallized from methanol, m. p. 210–212°; there was no depression in melting point when mixed with an authentic sample of jatrorrhizine iodide, m. p. 210–212°.

The Filtrate.—The filtrate after the separation of the hydrochloride was treated with ammonia and a precipitate was obtained. This was taken up in ether, and the residue on removal of ether melted at 205–206°, dec. It appeared to be an impure tetrahydrojatrorrhizine which had been previously obtained from the above hydrochloride.

The samples of root, stem, and bark of both young and old plants of *M. acanthifolia*, collected at different times of the year were examined chemically by the above method of separation of the alkaloids and the results are given in Table II. It is seen that the per cent of alkaloids is highest in the root of old plants which were harvested during the winter.

SUMMARY

1. Morphological differences of *Mahonia acanthifolia* Dox and *M. serpentaria* D. C. have been noted.

2. In both species of *Mahonia*, the alkaloids are distributed in cells of the different parts of the plant.

3. It is shown that the root of *M. acanthifolia*, when collected in winter, contains the highest per cent of the alkaloids, e.g., oxyacanthine in traces, berberine (0.5 per cent), neoprotine (0.0068 per cent), palmatine (0.07 per cent), and jatrorrhizine (0.138 per cent).

4. It has been suggested that the isolation and identification of the alkaloids from these two species could be an additional support for them to be placed into separate *Mahonia* species.

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A Convenient Laboratory Preparation of Ethylmorphine Hydrochloride, U. S. P. XIII*

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A procedure is described for preparing ethylmorphine hydrochloride in 90 per cent yield.

THE SYNTHESIS of ethylmorphine hydrochloride meeting pharmacopeial standards involves:

(*a*) Ethylation of morphine.
(*b*) Isolation and purification of ethylmorphine, including removal of unconverted morphine.
(*c*) Conversion of ethylmorphine to its hydrochloride.
(*d*) Purification of the hydrochloride.
(*e*) Recovery and re-use of alkaloidal material left in mother liquors.

Step *a* has been effected by a variety of reagents. A brief summary of the more important ethylation methods reported before 1952 is given in Small's monograph (1); no new ones have appeared since that date. We have found that ethyl bromide, introduced for this purpose by Mering (2), is, in spite of its volatility (b. p. 38°), very satisfactory. We have used ethanolic potassium hydroxide in the reaction mixture rather than the more troublesome ethanolic sodium ethoxide.

A description of the details of the remaining operations (steps *b* to *e*) has not appeared in the patent literature. The process recommended by Schwyzer (3) involves proceeding from crude ethylmorphine to purified ethylmorphine and then converting the latter to its hydrochloride. (It is not indicated whether or not this hydrochloride is sufficiently pure to meet official specifications.) The procedure described below requires fewer steps; the hydrochloride produced passes all U. S. P. XIII tests.

The yield data that have been previously reported are incomplete. Mering (2) claims an 85–90 per cent yield of *crude* ethylmorphine. Schwyzer (3) states that 85–90 per cent of *purified* ethylmorphine is obtained, exclusive of the quantity of morphine recovered. However, our interpretation of the data obtained at Merck, Darmstadt (4), where, apparently, a process very similar to the one described by Schwyzer (3) was employed, indicates that the yield of satisfactory product was 68 per cent based upon the total morphine charged and 79.4 per cent based upon the net morphine consumed (morphine charged minus morphine recovered). In several series of experiments, each comprising three successive runs, we have obtained a minimum yield of 90 per cent of final product based upon net morphine consumed. The data for one such series are given in Table I.

EXPERIMENTAL

A saturated solution of potassium hydroxide pellets, U. S. P., in absolute ethanol[1] was prepared in advance and assayed, by titration, for alkali concentration.

Ethylation.—One of the side necks of a 2-liter three-necked flask was fitted with a Claisen type adapter.[2] The assembly was now provided with a mercury-sealed stirrer, a 24-inch condenser protected by a calcium chloride tube, a thermometer to read the internal temperature of the reaction mixture and a 500-cc. dropping funnel. In the flask were placed 44.54 Gm. (0.156 mole) of anhydrous morphine,[3] 0.2 Gm. of zinc dust, 0.4 Gm. of anhydrous sodium sulfite, and 600 cc. of absolute ethanol.[4] Then sufficient ethanolic potassium hydroxide was added to provide 0.168 mole (an 8% excess) of alkali.[5] The mixture was stirred at room temperature until virtually all the morphine had dissolved. A solution of 20.42 Gm. (0.188 mole, a 20% excess) of ethyl bromide in 100 cc. of ethanol[1] was added rapidly. The mixture was heated under reflux for three hours.

Change of Solvents.—The reaction mixture was allowed to cool to about 50°, the condenser was set for distillation, and heating was resumed. In the course of about one and a half hours 1165 cc. of warm dry toluene[6] was allowed to run into the flask while simultaneously a mixture of alcohol and toluene was distilled out. The displacement of alcohol by toluene was considered to be complete when the vapor temperature reached 90°. By the time 1165–1170 cc. of distillate had been collected.

Recovery of Morphine.—The reaction mixture was cooled to 60–80° and transferred to a separatory funnel. It was shaken with two 40-cc. por-

* Received June 12, 1950, from the Research Laboratories of the New York Quinine and Chemical Works, Inc., Brooklyn, N. Y.

† The suggestions and criticisms of Dr. W. C. Bywater, S. B. Penick and Co., in the preparation of this manuscript are gratefully acknowledged.

[1] Denatured alcohol, Formula 3-A, was used.

[2] Ace Glass Inc., Catalog "40," item 4856.

[3] The sample used weighed 50.0 Gm. and was 94.90% pure.

[4] E.g., 45.3 Gm. of absolute potassium hydroxide assaying 3.707 milliequivalents of KOH per gram of solution.

[5] In subsequent runs of a series, part or all of this toluene contains recovered ethylmorphine.

tions of 5% aqueous potassium hydroxide and then with 100 cc. of water. The combined aqueous solutions were extracted once with 25 cc. of toluene; the latter was added to the main toluene solution.

The aqueous solution was acidified with acetic acid, boiled to remove traces of toluene, charcoaled, and filtered. The filtrate was cooled in an ice bath. Morphine was precipitated by the dropwise addition, with stirring, of concentrated ammonia to a *p*H of about 10.

Isolation and Purification of Ethylmorphine.—The toluene solution at 60–80° was shaken with three 165-cc. portions of 0.5 *N* H_2SO_4 and with one 25-cc. portion of water. The combined aqueous solutions were brought to *p*H 5. Three grams of $NaHSO_3$ was added and the solution was concentrated to one-half its original volume. During the final one-half hour of heating, 2 Gm. of Nuchar was also included. The solution was filtered hot, placed in a separatory funnel, cooled to 60–80° and covered with 150 cc. of toluene. Forty per cent sodium hydroxide solution was added, with intermittent shaking, until the *p*H was about 10. The layers were separated; the alkaline solution was extracted twice more with 100-cc. portions of toluene. The combined toluene solutions[6] were washed once with 25 cc. of water and then dried over anhydrous Na_2SO_4.

Precipitation of Crude Ethylmorphine Hydrochloride.—The filtered toluene solution[7] was chilled to 10°. A 10-cc. portion was removed and set aside. The remainder was stirred vigorously while to it was added a few drops of a solution of 12 cc. concentrated HCl in 400 cc. of dry acetone. The addition was interrupted and the oily precipitate was rubbed until it crystallized. Then the dropwise addition of the HCl solution was resumed and continued until the mixture became slightly acid to Congo red paper. The 10 cc. of solution that had been set aside was now stirred in.[8]

The mixture was refrigerated at 10° overnight. The crystals were removed by filtration and slurry-washed twice with 150 cc. of an acetone-toluene mixture (8 parts acetone; 5 parts toluene by volume). The washings were combined with the mother liquor. The crude ethylmorphine hydrochloride[9] was air-dried in the dark.

Recovery of Ethylmorphine.—The acetone-toluene solution was distilled at atmospheric pressure through an 8-inch Vigreux column in order to strip the acetone. This operation was considered to be complete when the vapor temperature reached 90°. The toluene solution was cooled to 60–80° and shaken with 100 cc. of 10% NaOH. The toluene layer is used in a subsequent run in lieu of a portion of the toluene needed to displace the alcohol at the conclusion of the ethylation.

Yield Data.—Table I summarizes the data obtained in three successive runs.

Table II presents the same data in terms of a materials balance for the three runs as a unit.

Ethylmorphine Hydrochloride, U. S. P. XIII.—Ten grams of crude ethylmorphine hydrochloride (from Run C) was dissolved in 40 cc. of boiling water and made slightly acidic to Congo red paper by the addition of a few drops of 3% HCl. Then 0.05 Gm. of $NaHSO_3$ and 0.2 Gm. Nuchar were added. The mixture was boiled for one-half hour—during which time the volume was allowed to decrease—and filtered hot. The charcoal was washed with a small

[6] When the toluene solution had a purplish cast, as happened occasionally, it was also washed once with a dilute solution of sodium hydrosulfite.

[7] In subsequent runs of a series a toluene solution containing recovered ethylmorphine was added at this point.

[8] The *p*H of 0.5% solutions of several lots of commercial ethylmorphine hydrochloride was found (M. Russo, N. Y. Q. Control Laboratory) to lie between 5.3 and 5.7.

[9] In a number of cases this "crude" product satisfied U. S. P. purity requirements. A recrystallization is recommended, however, in order to improve the color stability and to insure uniform acceptability.

TABLE I.—ETHYLATION OF MORPHINE: YIELD DATA

Run	Morphine Input: Gm.	Morphine Input: A. M. A.[a] Content,[b] %	Crude Ethylmorphine Hydrochloride: Solid, Gm.	Crude Ethylmorphine Hydrochloride: Ethylmorphine Content,[c] %	Crude Ethylmorphine Hydrochloride: In Mother Liquor as Alkaloid, Gm.	Recovered Morphine: Solid, Gm.	Recovered Morphine: A. M. A. Content,[d] %	Recovered Morphine: In Mother Liquor as A. M. A.[a]
A	46.93	44.54	40.32	32.30	Into B	9.05	7.60	0.20
B	46.93	44.54	45.73	35.67	Into C	7.97	6.42	0.53
C	46.93	44.54	48.03	38.45	1.87	8.85	7.25	0.07

[a] A. M. A. = anhydrous morphine alkaloid.
[b] Assays by N. Y. Q. Control Laboratory.
[c] Duplicate assays by E. S. Ellner and Walter Smith.
[d] Assays by N. Y. Q. Plant Laboratory.

TABLE II.—ETHYLATION OF MORPHINE: MATERIALS BALANCE[a]

Input		Output	
Morphine	133.6 Gm. (100%)	As solid crude ethylmorphine hydrochloride	90.84 Gm. (72.48%)
		As recovered ethylmorphine	1.67 Gm. (1.25%)
		As recovered morphine (solid)	21.28 Gm. (15.93%)
		As recovered morphine (in solution)	0.80 Gm. (0.60%)
		Loss	13.0 Gm. (9.7%)
		Total	133.6 Gm. (100%)

[a] All calculations expressed in terms of anhydrous morphine.

volume of boiling water, the washings were added to the filtrate. The *p*H was re-adjusted. The solution was stirred and chilled to induce rapid crystallization, and finally refrigerated for fifteen hours at 5–10°. The crystals were removed by filtration and slurry-washed with 10 cc. of ice water. After air-drying in the dark, the ethylmorphine hydrochloride (7.12 Gm.) met all U. S. P. XIII purity tests.

A materials balance made around this recrystallization step indicated that there had been no loss of alkaloid.

The ethylmorphine hydrochloride mother liquor is made alkaline and extracted with three portions of toluene. The toluene solution is added, in a subsequent run, to the solution from which crude ethylmorphine hydrochloride is to be precipitated.

SUMMARY

A procedure is described in detail for ethylating morphine with ethyl bromide in alcoholic medium. The replacement of ethanol by toluene at the conclusion of the ethylation, permits ready removal of unchanged morphine and further purification of the ethylmorphine in few steps. The addition of an acetone solution of hydrochloric acid to a toluene solution of ethylmorphine precipitates the alkaloid hydrochloride, which, as such, or after one recrystallization from water, meets U. S. P. XIII purity standards.

In a series of consecutive runs involving appropriate recycling of material, the yield of ethylmorphine hydrochloride, based upon morphine that has entered the reaction, is 90 per cent.

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Toxicological Studies on Synthetic Glycerin*

By ROBERT C. ANDERSON, PAUL N. HARRIS, and K. K. CHEN†

Acute and prolonged studies have failed to show any differences in the toxicity of natural and synthetic glycerin. The acute toxicities in mice following intravenous and oral administration are reported. Pathological changes in rats are compared after treatment for six months with natural and synthetic glycerin.

GLYCERIN, a trihydric alcohol, was discovered by Scheele in 1783 but was not used in pharmacy until many years later when it was employed as an emollient for retaining moisture on surfaces having a tendency to dry and crack. It first appeared in the third edition of the United States Pharmacopoeia, which became official in 1850. Today the use of glycerin is widespread and millions of pounds are consumed annually in the pharmaceutical industry alone. Lesser (1) has reviewed the many formulas where it is employed. Other uses include the manufacture of cigarettes, cosmetics, explosives, liqueurs, printing inks, liquid soaps, and lubricants.

Glycerin has been obtained for the most part as a by-product from the manufacture of soaps and fatty acids. During the war when large quantities were essential in the manufacture of explosives, other glycols were substituted in pharmaceuticals where possible. None seemed to be as universally acceptable as glycerin. It now appears that the use of detergents is increasing and if the manufacture of soaps declines, the supply of natural glycerin may become inadequate. The development of a process on a commercial scale for the production of synthetic glycerin will alleviate the necessity of finding substitutes.

Before synthetic glycerin can be used in products designed for human consumption, it must be shown to contain no harmful impurities. The toxicity of natural glycerin has been studied by numerous workers including Deichmann (2), von Oettingen (3), and Holck (4). Hart (5) could find no difference in the toxicity of natural and synthetic glycerin following oral administration of single doses to rats. We have attempted to compare the acute and chronic toxicities of the two products.

* Received May 5, 1950, from the Lilly Research Laboratories, Indianapolis 6, Ind.
Presented to the Scientific Section, A. Ph. A., Atlantic City meeting, May, 1950.
† The authors wish to acknowledge the assistance of Marian Ellaby, Nila Maze, John Owen, John S. Wells, and Harold Worth in these studies.

EXPERIMENTAL

The synthetic glycerin used in our studies was supplied by the Shell Chemical Corporation. It was a clear, colorless, syrupy liquid having a sweet taste, a slight characteristic odor, and was neutral to litmus. Other physical properties follow:

Specific gravity, 20/20°	1.2631
Purity, % by weight	99.80
Total chlorine, % by weight	0.009
Water, % by weight	0.30
Ash, % by weight	0.0004

The natural glycerin used was a pooled sample from various suppliers, all lots being U. S. P. quality or better.

Acute Toxicity in Mice.—For intravenous studies, 30% solutions by weight were prepared. This gave an injectable material which could be measured conveniently within practical limits. For oral studies, undiluted samples were administered by stomach tube. All animals were observed for one week and the LD_{50} for each study was computed by the method of Bliss (6). The results are found in Table I. All deaths occurred soon after intravenous injection but were somewhat delayed following oral administration. In the latter case, some mice died within ninety minutes and all died within forty-eight hours after ingestion. Obviously, no statistical difference can be shown between the toxicities of the two products.

Chronic Toxicity.—Fifteen female rats, six to eight weeks old, were housed in individual cages in temperature-controlled quarters. All were allowed to eat ground rat ration *ad libitum*. Five animals, which served as controls, were given tap water to drink; a second group of five received tap water containing natural glycerin 5% by volume, while the third group received the same concentration of synthetic glycerin as drinking water. All rats were weighed at weekly intervals. Hemoglobin was determined, and erythrocytes, leucocytes, and differentials were counted monthly for a period of six months. The animals were then sacrificed and submitted to necropsy. Both gross and microscopic studies were made of the heart, lungs, liver, spleen, stomach, intestines, kidneys, thymus, thyroid, and adrenals.

Effect on Growth.—The growth curves for each group are found in Fig. 1. The rate of growth in the control group is typical of female rats of the same strain and has been checked several times. When the body weight reaches 200–225 Gm., the gain levels off. Both groups which received 5% glycerin in the drinking water gained weight to about the same degree as the controls.

Effect on Hemoglobin.—The mean hemoglobin values determined as acid hematin by the Fisher electrohemometer are found in Table II. The figures are quite comparable to those found for the controls.

TABLE II.—AVERAGE HEMOGLOBIN VALUES IN PER CENT (15.6 GM./100 CC. EQUALS 100%)

Date	Control Group	Natural Glycerin	Synthetic Glycerin
3-21-49 (Normal)	87.8	90.6	79.2
4-18-49	101.2	108.2	104.8
5-24-49	114.8	110.2	109.0
7- 5-49	113.8	110.8	109.2
8- 2-49	113.6	104.6	96.8
8-25-49	109.8	104.6	106.3
9-27-49	112.6	109.8	105.3

Effect on Red Blood Cells.—The mean erythrocyte count for each group is found in Table III. Here, also, no variation from the control group is found, the number in general rising from the initial value recorded prior to treatment.

Effect on White Blood Cells.—The mean number of leucocytes is found in Table IV. An increase is noted at times, which is true in all groups. It has previously been found that rats, subjected to fre-

TABLE I.—ACUTE TOXICITY OF GLYCERIN IN MICE

Mode of Administration	Lot Used	Dose	No. Died/No. Used	LD_{50} ± S. E.
		Gm./Kg.		
Intravenous	Natural	3.75	1/10	4.37 ± 0.14 Gm./Kg.
		4.20	5/10	
		4.80	6/10	
		5.40	10/10	
	Synthetic	3.75	2/10	4.25 ± 0.16 Gm./Kg.
		4.20	5/10	
		4.80	8/10	
		5.40	9/10	
		Cc./Kg.		
Oral	Natural	16.0	1/10	20.63 ± 0.47 Cc./Kg.
		18.0	4/10	
		20.0	5/15	
		22.5	7/10	
		25.0	8/10	
		30.0	5/5	
	Synthetic	16.0	1/10	20.81 ± 0.58 Cc./Kg.
		18.0	2/10	
		20.0	6/15	
		22.5	7/10	
		25.0	8/10	
		30.0	5/5	

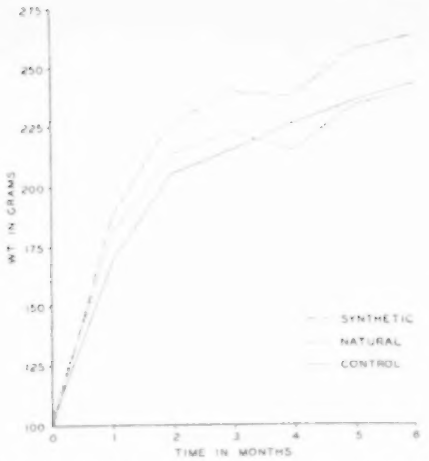

Fig. 1.—Effect on growth of rats receiving glycerin (5%) in the drinking water.

quent bleedings from the tail, often develop a mild leucocytosis.

Although differential counts were made monthly on all groups, no differences were found among the three groups that would justify the use of the necessary space for the voluminous records.

Pathological Findings: Control Group.—All rats were normal, grossly and microscopically.

TABLE III.—AVERAGE RED BLOOD COUNT (IN MILLIONS)

Date	Control Group	Natural Glycerin	Synthetic Glycerin
3-21-49 (Normal)	7.92	7.95	7.37
4-18-49	8.28	7.38	7.25
5-24-49	9.87	8.79	9.32
7-5-49	7.95	8.18	7.75
8-2-49	9.95	9.64	8.77
8-25-49	9.24	8.51	8.78
9-27-49	9.63	9.48	8.99

Natural Glycerin.—Grossly, all were normal except for a small thymus in two. One rat had a slight focal interstitial pneumonia not attributable to the drug. All microscopic sections were normal except those of the kidneys. In three out of the five, several tubules near the junction of the cortex and medulla contained calcified masses.

Synthetic Glycerin.—One rat died after four months. It was emaciated, the liver was small and moderately congested; the spleen was small and the thymus was atrophic; the stomach and intestines were empty. Microscopic examination of the spleen revealed small lymph nodules and moderate deposits of hemosiderin in reticular cells. It seems improbable that these changes were due to synthetic glycerin, for similar ones were not found in the other animals. Except for the kidneys, the tissues of all other rats were grossly and microscopically normal. As with natural glycerin, three out of the five rats had calcified masses in the tubules near the junction of the cortex and medulla.

TABLE IV.—AVERAGE WHITE BLOOD COUNT (IN THOUSANDS)

Date	Control Group	Natural Glycerin	Synthetic Glycerin
3-21-49 (Normal)	17.4	15.9	17.2
4-26-49	28.1	24.3	23.6
5-24-49	18.4	20.7	23.2
6-21-49	20.6	24.5	20.2
7-19-49	20.0	16.0	16.1
8-23-49	14.4	17.2	17.7
9-27-49	17.2	16.8	16.8

CONCLUSIONS

1. No difference has been shown between the toxicity of natural and synthetic glycerin following intravenous and oral administration to albino mice.

2. Rats that received 5 per cent glycerin (either natural or synthetic) as drinking water for six months gained weight comparable to that of the controls. No change was found in the hematopoietic system and the mild damage to the kidneys occurred with glycerin from both sources. No other visceral damage was apparent.

3. Synthetic glycerin appears to be as harmless as natural glycerin to laboratory animals. However, additional work may be necessary before it is used in pharmaceutical products for human consumption. Since large quantities serve in the manufacture of cosmetics, prolonged studies by local application must be also completed before its use in this industry may be justified.

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Cultivation Studies of the Solanaceous Drugs

II. The Effect of Nutritional and Soil Reaction Fertilizers on the Production Yields and Total Alkaloidal Content of *Atropa belladonna* and *Hyoscyamus niger**,†

By W. R. BREWER‡ and L. DAVID HINER§

In order to clarify some of the contradiction and confusion existing in the literature concerning the value of adjusting *p*H, and nitrogen, phosphorus, and potassium levels in solanaceous plants and their alkaloid production, a study was begun with belladonna and hyoscyamus plants growing in controlled variations of these elements and their concentrations. The results of these initial studies were subjected to statistical interpretation to determine their significance.

The recent national emergency provided evidence that at certain times, the United States has a definite economic need for products from plants of the *Solanaceae* family. In turning to the literature on the culture of these plants, producers found the reports both contradictory and inadequate in detail. Many conclusions were found to be drawn without sufficient basis. Few reports gave evidence of statistical analysis. Production yields were frequently stated without designating a fresh weight or dry weight basis. In commenting on this last condition, Mothes (1) emphasized the necessity for more precise data relative to production of experimental material, more particularly as to its "physiological condition." In the U. S. D. A. Bulletin No. 663, Stockberger (2) in outlining belladonna cultivation stated that propagation descriptions lacked details.

Great variations of opinion were found in the literature concerning the value of applying nutritional and soil reaction fertilizers in alkaloid-bearing plant production. Following are illustrations of such diversity:

Chevalier (3).—Phosphorus and potash manures increased the content of alkaloid in belladonna, but the highest yield (0.68 per cent to 0.75 per cent) was produced by using nitrogen fertilizer. Similar results were obtained with other solanaceous plants.

Ploski (4).—The alkaloid content of *Datura stramonium* is independent of nitrogen and potassium fertilization. Alkaloids increase only with size and age of leaves.

Treven and Schreiber (5).—Failure of nitrogen and phosphorus in nutrient medium caused a decrease in alkaloids in belladonna. Alkaloids increased when potassium was present in reduced amounts only.

Ransom and Henderson (6).—Of 11 belladonna plots, variously manured, the highest alkaloidal yield was obtained with no manure but with full sunlight. Alkaloids were not materially increased, but the yield of green plant per acre was largely increased.

Koch (7).—Sodium nitrate and ammonium sulfate increase growth from 33 per cent to 50 per cent in belladonna.

Johnson and Nunez-Melendez (8).—Commercial fertilizers increase the alkaloidal content of wild stramonium, but not as much as cultivation alone.

Sandfort (9).—Those factors that produce the best growth favored the best production of alkaloids in stramonium.

Sukhoryakov and Borodulina (10).—Acidification of medium decreases alkaloidal content in stramonium.

Stillings and Laurie (11).—Fertilization of belladonna does not increase alkaloid yield significantly, but *p*H 6 in media increased alkaloids over *p*H 7.

McNair (12).—With decrease in *p*H value of soil there is a definite decrease in the percentage of plant genera producing alkaloids. With plant families producing alkaloids, the maximum molecular weights of the alkaloids decrease with a decrease in soil *p*H.

Schneider (13).—The alkaloid yield is higher in belladonna if the soil is heavily limed.

In order to clarify some of the confusion in the literature, studies were begun in Ohio with authenticated individuals of the species, *Atropa belladonna* in 1942 and 1943, and *Hyoscyamus niger* (biennial) in 1947, growing under varied conditions of soil nutrition and *p*H.

* Received August 3, 1948, from the College of Pharmacy, University of Utah, Salt Lake City.
Presented to the Scientific Section, A. Ph. A., Atlantic City meeting, May, 1950.
† Part of a dissertation submitted to the Graduate School, Ohio State University, in partial fulfillment of the requirements for the degree of Doctor of Philosophy.
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EXPERIMENTAL

In 1942 and 1943, the belladonna plants were set out in the field during the last week in May and first week in June and three harvests were obtained. However, due to the unusually cold, wet spring of 1947 in Ohio, the hyoscyamus plants were set out in the second week in June and only two harvests were obtained. For purposes of replication of these tests, the belladonna plots in 1943 were planted in three different garden locations in and around the University. A complete test was also included involving two-year-old plants. The hyoscyamus was planted in 1947 and no replications were made.

All plants used in these tests were set in plots of 50 each and spaced 12 in. by 12 in. Each replication contained 16 plots with varied nutritional and *p*H levels and one control plot. The soil nutrients and the two levels at which each was maintained were:

Nitrogen—High (H): 50-100 parts per million (p.p.m.).
Low (L): 10-50 p.p.m.
Phosphorus—H: 5-10 p. p. m.
L: 1-3 p. p. m.
Potassium—H: 20-40 p. p. m.
L: 5-20 p. p. m.

The two *p*H levels maintained were:

H: *p*H 6.5 to 7.5
L: *p*H 5.5 to 6.5

The arrangement of these nutritional and *p*H levels in the 16 plots was as follows:

Plot No.	Treatment			
	N	P	K	*p*H
1	L	L	L	H
2	L	L	L	L
3	H	H	H	H
4	H	H	H	L
5	L	H	H	H
6	L	H	H	L
7	L	L	H	H
8	L	L	H	L
9	H	L	L	H
10	H	L	L	L
11	H	L	H	H
12	H	L	H	L
13	L	H	L	H
14	L	H	L	L
15	H	H	L	H
16	H	H	L	L

During the first two weeks' growth in the field, and every fourteen to twenty days thereafter, a well mixed, representative sample of the soil of each plot was tested for the content of each of the three nutrients and for the *p*H level. The method of testing soils for N, P, and K was taken from the manual, *Soil Testing*, by C. H. Spurway, Michigan State College, Agricultural Experiment Station, East Lansing, Mich. This procedure, using rapid colorimetric tests on a filtered aqueous soil extract indicates, in parts per million, the total available (water-soluble) nitrates, phosphates, and potassium in mineral combination. Testing the *p*H of the soil followed the "Soiltex" method of the Edwards Laboratory of East Lansing, Mich.

The spaces provided for nutritional tests were previously cultivated, clean plots. Consequently, soil nutrients were found to be low. Additions of mineral salts were made when necessary, as indicated by soil tests, to maintain the levels prescribed for each plot. Minerals used to increase the soil nutrients were ammonium nitrate, calcium phosphate and potassium chloride. These were applied at the rate of from 1 to 4 lb./100 sq. ft. by spreading uniformly over the surface of the plot and "chopping" the mineral into the soil with a hoe. Care was exercised in preventing contact of the salts with the plants themselves. This treatment provided sufficient nutrients so that applications were rarely needed more than two or three times in a growing season. The high potassium levels were the most difficult to maintain probably due to leaching from the soil. In order to include any soil reaction provided by adding minerals, *p*H tests followed the nutrient additions and the necessary minerals were then added to adjust the *p*H without disturbing the desired nutritional level. The soils were normally slightly acid (average about 6.8). Sulfur was used for maintenance of the lower *p*H level and lime for the higher level. These were applied in the same manner as the nutrients were applied.

Harvests were made as often as warranted by plant growth. Three cuttings were taken from first-year belladonna plants, but only two from the first-year hyoscyamus plants because of the shortened growing season in 1947. Belladonna was harvested by cutting the stems about 6 to 8 in. above the ground, leaving several leaves intact to maintain the vitality of the plants until new shoots and leaves developed. After determining the fresh weight of the crop, the cut stems were impaled upon nails protruding through a strip of pine wood 1 in. by 2 in. by 15 ft. This "rack" was carried to a drying loft, fitted on supports, and left to dry. After drying, the belladonna leaves and tops were removed from the racks, weighed, and stored in paper bags until ground to a moderately coarse powder for assay.

Hyoscyamus was harvested by cutting all but six of the lowest leaves of the first-year rosette. The crop was weighed in the fresh condition, carried to the drying loft, and spread thinly on cloth-bottom trays to dry. The leaves were weighed when dry and placed in paper bags until ground to a moderately coarse powder for assay.

For each sample obtained in this manner, a record was kept of the name of the plant, number of plants producing the harvested material, the fresh weight, and the dry weight. When assayed, the result was also recorded. From this information, the yield in air-dried weight produced per plant during the season was determined. The annual yield in pounds of air-dried weight per acre was calculated, based on the fact that a spacing of 12 in. by 12 in. allows for 37,180 plants per acre when every seventh row is left vacant for cultivation purposes.

The data concerning belladonna obtained in this experiment are given in Table I, the upper figures being the total annual air-dried weight in pounds per acre, and the lower figures, the alkaloidal assay in parts per 10,000, both representing leaves and tops. All assays reported in this experiment were obtained by analyses carried out according to the method of the U. S. P. XII.

TABLE I.—BELLADONNA PRODUCTION AND ALKALOIDAL ASSAYS

Plot	1942	Replications—1943 (3 Gardens): No. 1	No. 2	No. 3	Mean
1	3312	2326	1728	1654	2255.0
	.36	.58	.44	.46	.460
2	3254	2522	2002	1990	2606.0
	.41	.51	.42	.48	.455
3	5981	4398	3317	2825	4130.2
	.30	.60	.38	.48	.440
4	5712	3702	2801	2522	3684.2
	.29	.66	.43	.32	.425
5	5203	1974	2383	1564	2781.0
	.29	.62	.40	.35	.415
6	4800	876	2947	1548	2317.8
	.33	.62	.38	.44	.442
7	3532	1024	2645	2221	2355.5
	.33	.56	.47	.39	.438
8	4041	1884	1892	1736	2388.2
	.33	.64	.41	.38	.440
9	3897	696	2235	2170	2249.5
	.28	.60	.37	.38	.408
10	6700	2580	2080	2383	3435.8
	.41	.50	.35	.43	.422
11	4473	2228	3087	2240	3008.0
	.29	.63	.37	.38	.418
12	4665	2203	4177	3472	3629.2
	.36	.67	.37	.45	.462
13	2918	2571	2023	1736	2312.0
	.35	.66	.36	.39	.440
14	4406	925	1960	1892	2303.2
	.41	.70	.48	.38	.508
15	6038	2776	2449	2272	3378.8
	.28	.60	.45	.35	.420
16	5366	2080	4398	2653	3624.2
	.28	.62	.34	.39	.408
Mean:	4643.4	2172.8	2619.6	2178.9	2903.7
	.331	.614	.401	.403	.438

NOTE.—The upper figure in each pair of plot data represents *pounds* and lower figure *per cent*.

The data of Table I were interpreted with the aid of the Statistics Laboratory, Ohio State University, in two analyses of variance in five-way classifications. The classifications were:

Level of nitrogen
Level of phosphorus
Level of potassium
*p*H level
Replication

This treatment of the data makes possible testing of several different kinds of hypotheses, those tested in this study pertaining to "main effects" and "interactions" between two classifications. For each level (high or low) of each classification, a "main effect" is defined by:

(Mean of level of classification) minus (Grand mean)

Table II shows the mean dry weight yields for the "high" level of each soil treatment classification, as well as the combined means of treatments at "high" levels in two classifications.

It may be noted that if the mean of the "high" level of a given classification is greater than the grand mean, then the mean of the "low" level of the same classification will be less than the grand mean by the same amount, so that the sum of the two main effects for each classification is zero.

Interactions between two classifications are a measurement of any effect the combination of the levels of the classifications may have in addition to the general effect of the levels of the classifications separately. Thus the "high-high" interaction term between two classifications would be given by

(the ("Hi-Hi" combination mean) minus ("Hi" level main effect of first classification) minus ("Hi" level main effect of second classification) minus (Grand mean).

Considering only the four soil treatments, there are just six pairs of classifications, and for each pair there are four interaction terms: "Hi-Hi,"

TABLE II.—MEAN YIELDS OF CLASSIFICATIONS N, P, K, AND *p*H IN BELLADONNA PRODUCTION TEST

	N	P	K	*p*H
Mean yield of "Hi" level	3392.5	3066.4	3036.8	2808.8
Mean yield of combination (Hi-Hi level):				
P	3704.4			
K	3612.9	3228.3		
*p*H	3191.6	3150.5	3068.7	
Replications means:				
No. 1—1943 Plots			2172.8	
No. 2—1943 Plots			2619.6	
No. 3—1943 Plots			2178.9	
Average 1942 Plots			4643.4	
Grand mean:			2903.7	

"Hi-Lo," "Lo-Hi," and "Lo-Lo." Finally of these four terms, the sums

"Hi-Hi" plus "Hi-Lo"
"Hi-Hi" plus "Lo-Hi"
"Lo-Lo" plus "Hi-Lo"
"Lo-Lo" plus "Lo-Hi"

are all zero, so that given any one of the four terms, the others are equal to it in numerical value. The main effect and interaction terms corresponding to the means shown in Table II are given in Table III.

TABLE III.—EFFECTS OF HI-LEVEL CLASSIFICATIONS N, P, K, AND *p*H IN BELLADONNA PRODUCTION TEST

	N	P	K	*p*H
Main effect	+488.8	+162.7	+133.1	−94.9
Interaction with:				
P	+149.2			
K	+87.3	+28.8		
*p*H	−106.0	+179.0	+126.8	
Replication main effect:	1943 Plots No. 1			−0 730.9
	1943 Plots No. 2			−0284.1
	1943 Plots No. 3			−0724.8
	1942 Plots			+1739.7

For each of the entries in Table III, the hypothesis was tested that *in the population consisting of all possible repetitions of the experiment, the qualities of which these entries are a sample, are zero.* As a result of the test, the probability of obtaining as numerically large a value as was obtained is computed on the basis of the stated hypothesis. These probabilities are indicated in the right-hand column of Table IV, which also shows the final calculations leading to them.

Reference to Table IV shows that the main effect of the nitrogen fertilizer is very significantly different from zero, and reference to Table III shows that the high level of nitrogen increased the mean yield; hence, the conclusion may be drawn that adding nitrogen fertilizer increases yield. Tables III and IV also show evidence of another effect which was entirely unnoticed prior to statistical analysis, namely that interaction between high phosphorus and high *p*H significantly increases belladonna production. The 1% significance of replications shown in Table IV indicates that the results of each treatment in the production tests with belladonna agreed very significantly throughout all replications. Addition of phosphorus to the high level in soils was found to be "almost" significant. Further experimentation is necessary to establish the value of this fertilizer element in belladonna production. In Table III, the main effect of Hi *p*H is shown to be a negative quantity. The Lo *p*H level would therefore be a positive quantity of the same numerical value. This indicates a tendency toward an increase in production of belladona with the low *p*H level, but Table IV reveals that it is remote from significance.

The technique of analysis of data on dry weight yield was applied in the same manner to the alkaloidal assays of the same plants. Table V shows the values of the main effect and interaction terms, in alkaloidal assay test corresponding to Table III which concerned dry weight production.

Corresponding tests of significance were made, the results of which are shown in Table VI. Here, the combined effect of high levels of nitrogen and potassium is to raise the assay significantly, while nitrogen alone tends to decrease the assay almost significantly. The replications again agreed very significantly in their results.

TABLE V.—EFFECTS OF HI-LEVEL CLASSIFICATIONS N, P, K, AND *p*H IN BELLADONNA ALKALOID TEST

	N	P	K	*p*H
Main effect	−1.22	−0.03	−0.25	−0.78
Interaction with:				
P	−0.19			
K	+1.34	−0.41		
*p*H	+0.38	−0.06	+0.03	
Replication main effect:	1943 Plots No. 1			+17.69
	1943 Plots No. 2			−3.62
	1943 Plots No. 3			−3.44
	1942 Plots			−10.60

TABLE IV.—SIGNIFICANCE OF VARIATIONS IN BELLADONNA PRODUCTION TEST

Source of Variation	Sums of Squares	D. F.	Mean Square	F	Significance
Interactions between:					
N and P	1,422,951	1	1,422,951	3.13	None
N and K	488,077	1	488,077	1.07	None
N and *p*H	718,469	1	718,469	1.58	None
P and K	52,958	1	52,958	0.12	None
P and *p*H	2,050,266	1	2,050,266	4.51	5%
K and *p*H	1,029,464	1	1,029,464	2.26	None
Main effects of:					
N	15,292,988	1	15,292,988	33.64	1%
P	1,685,113	1	1,685,113	3.71	Almost
K	1,133,959	1	1,133,959	2.49	None
*p*H	576,650	1	576,650	1.27	None
Replications	66,667,710	3	22,222,570	48.89	1%
Random	22,729,059	50	454,581		
Total	113,847,664	63			

For 1 and 30 D. F.: $F_{5\%} = 4.00$; $F_{1\%} = 7.47$.

TABLE VI.—SIGNIFICANCE OF VARIATIONS IN BELLADONNA ALKALOID TEST

Source of Variation	Sums of Squares	D. F.	Square	Mean F	Significance
Interactions between:					
N and P	2.25	1	2.25	0.09	None
N and K	115.56	1	115.56	4.52	5%
N and pH	9.00	1	9.00	0.35	None
P and K	10.56	1	10.56	0.41	None
P and pH	0.25	1	0.25	0.01	None
K and pH	0.06	1	0.06	0.00	None
Main effects of:					
N	95.06	1	95.06	3.72	Almost
P	0.06	1	0.06	0.00	None
K	4.00	1	4.00	0.16	None
pH	39.06	1	39.06	1.53	None
Replications	7211.12	3	2403.71	94.12	1%
Random	1277.00	50	25.54		
Total	8763.98	63			

The analysis of the data from the 1947 hyoscyamus experiments differs from the belladonna since there were no replications. Table VII shows the dry weight and alkaloidal assays of the hyoscyamus plots.

TABLE VII.—PRODUCTION AND ALKALOIDAL ASSAYS OF *Hyoscyamus niger* (BIENNIAL) IN NUTRITION AND pH TEST PLOTS

Treatment (Plot No.)	Lb./Acre Yield	Assay Percentage
1	8208	.08
2	2928	.09
3	9720	.11
4	6736	.08
5	4446	.07
6	3064	.07
7	5094	.08
8	4491	.07
9	5655	.06
10	5643	.10
11	5615	.08
12	5625	.07
13	4923	.07
14	7816	.06
15	6003	.10
16	4792	.10

The same calculations and tests were made on each of the sets of measurements in Table VII as were described in the discussion concerning belladonna. Nothing approaching significance was found in the production yield data for hyoscyamus, and therefore, no information on those calculations is included. Further study is needed to determine the value of fertilizers and pH control in hyoscyamus production.

For the consideration of the value of such treatment on alkaloidal content, however, Table VIII is offered showing the main effect and interaction terms in the analysis of the assays.

Table IX, containing the results of the test for significance of the effects of the nutrition and pH

TABLE VIII.—EFFECT OF HI-LEVEL CLASSIFICATIONS N, P, K, AND pH IN *Hyoscyamus niger* (BIENNIAL) ALKALOID TEST

	N	P	K	pH
Main effect	−0.69	−0.19	+0.19	−0.06
Interaction with:				
P	+0.81			
K	−0.06	+0.19		
pH	−0.06	+0.44	+0.56	

TABLE IX.—SIGNIFICANCE OF VARIATIONS IN *Hyoscyamus niger* (BIENNIAL) ALKALOID TEST

Source of Variation	Sums of Squares	D. F.	F	Significance
Interaction between:				
N and P	10.5625	1	7.75	5%
N and K	0.0625	1	0.05	None
N and pH	0.0625	1	0.05	None
P and K	0.5625	1	0.41	None
P and pH	3.0625	1	2.25	None
K and pH	5.0625	1	3.72	None
Main effects of:				
N	7.5625	1	5.55	Almost
P	0.5625	1	0.41	None
K	0.5625	1	0.41	None
pH	0.0625	1	0.05	None
Random	6.8125	5		
Total	34.9375	15		

Mean square for random error: 1.3625.
Values of F for 1 and 5 D. F.: $F_{0.05} = 6.61$; $F_{0.01} = 16.26$.

treatments, in regard to assays, shows that only the interaction between N and P is significant, while the main effect of N alone is almost significant. It may be concluded, therefore, that the combination treatment of Hi N and Hi P significantly increases the alkaloidal content, but that N alone in high levels almost significantly tends to decrease alkaloidal content.

CONCLUSIONS

The results of the study show:

1. That high nitrogen fertilizer such as ammonium nitrate used alone is the best application for increasing dry weight production of belladonna leaves.

2. That a combined phosphorus and high *p*H fertilizer (calcium phosphate and lime) is second in value in its increasing of belladonna leaf production.

3. That the best treatment for increasing belladonna alkaloid production is a combined nitrogen and potassium fertilizer (ammonium nitrate and potassium chloride).

4. That, although no fertilizer was found which increased hyoscyamus production significantly, the increase of its alkaloidal content may be brought about by the use of a nitrogen and phosphorus combination fertilizer (ammonium nitrate and calcium phosphate).

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Investigation of *Chrysactinia Mexicana*, A. Gray*,†

By LEWIS LAPPAS and CARROLL B. GUSTAFSON‡

The authenticated, air-dried aerial portion of this heath-like shrub, with relatively few flower heads present, has been subjected to the usual proximate, qualitative, and quantitative analyses. No extraordinary constituents seem to be present. A sugar, maltose, was isolated from one series of extracts of the prepared drug. Less than one-half of one per cent of a volatile oil was separated from another extract. This oil contained a camphoraceous, ketone-like component. A separation by the method of Pincus, modified by Costello, yielded extracts which produced characteristic color reactions with *m*-dinitrobenzene. These results from the Zimmerman color reaction, modified by Pincus, were highly suggestive of 3- and/or 17-ketosteroids being present in the extracts. Some estrogenic activity from an extract of the drug was obtained on ovariectomized rats.

A CITIZEN of Boston, Mass., on one of his trips through Central America learned of a drug which enjoyed considerable popularity as a medicinal agent, and was reputed to produce a so-called "rejuvenation" of the male. Some time thereafter, small quantities of the plant were collected for investigation.

A survey of the literature revealed no report on chemical study of this plant. Inasmuch as estrogenic and androgenic principles have been detected and isolated from various plant drugs in recent years, this investigation was carried out in an attempt to contribute to the chemistry of this seemingly valuable drug.

HISTORY

In the year 1849 Asa Gray published a macroscopic description of the plant, *Chrysactinia Mexicana*, A. Gray, in the *Memoirs of the American Academy* ". . . Shrub, one or two feet high,

* Received May 5, 1950, from the Massachusetts College of Pharmacy, Boston.
Presented to the Scientific Section, A. PH. A., Atlantic City meeting, May, 1950.
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heathlike; etc. . . . It must therefore be referred to *Tagetineae*, where it combines the characters of the two divisions, having the rays and the style of the *Tageteae*, with nearly the pappus, involucre and foliage of porophyllum. The name is given in allusion to the (golden yellow) rays, which distinguish the plant from the porophyllum . . ." (1).

Chrysactinia Mexicana bears many synonyms. The following are representative, with their localities: Calanca (Vera Cruz); Yeyepaxtle (Puebla); San Nicolas; hierba de San Nicolas (Coahuila, Durango, San Luis Potosi); damiana, damianita (Chihuahua, Durango, Texas, etc.); mariola (Valley of Mexico); false damiana, garanona, romerillo (Coahuila, Hidalgo); also said to be known as guayule (2).

In Texas it is distributed in the plains country of the northwest, in Edward's Plateau, and in the Trans-Pesos area where it is known as "false damiana" (3).

The drug finds local use as a domestic medicinal agent and is said to be an aphrodisiac, antispasmodic, sudorific, diuretic, antipyretic, and antirheumatic (2).

EXPERIMENTAL

Preparation for Analysis.—The drug, in the form of the air-dried aerial portion of the shrub, was authenticated as true *Chrysactinia Mexicana*, A. Gray of the compositae family by Dr. Heber W. Youngken of the Massachusetts College of Pharmacy, Boston, Mass.

The garbled drug was pulverized to 20-mesh size, stray iron particles were removed by a magnet, and the whole thoroughly mixed. Samples for analysis were withdrawn from this uniformly mixed material. All quantitative determinations were calculated on the basis of the moisture-free drug.

Proximate Analyses—Moisture.—The average moisture content of two samples of the air-dried drug was 6.62% as determined by the "Moisture Method by Toluene Distillation" of the United States Pharmacopœia XI (4).

Ash Determinations.—The methods were those of the U. S. P. XI, and results were as follows:

Total ash	4.50%
Acid-insoluble ash	0.24%
Acid-soluble ash (by difference)	4.26%
Water-insoluble ash	2.32%
Water-soluble ash (by difference)	2.18%

Spectrographic Qualitative Analysis of the Ash.—This analysis made on the total ash by an industrial laboratory indicated that the elements sodium and potassium were present as major constituents. Calcium, magnesium, and iron were present in about 0.01% amounts, and silicon and aluminum were present in trace amounts.

Extractives.—Suitable, accurately weighed samples of the powdered drug were completely extracted by the methods of the U. S. P. XI (4) employing a Soxhlet continuous extraction apparatus with duplicate samples and alcohol, diluted alcohol (50% aqueous), distilled water, absolute ether, and petroleum benzin as the solvents.

Alcohol-soluble extract (20 hr.)	12.70%
Diluted alcohol-soluble extract	15.82%
Water-soluble extract	24.67%
Volatile ether-soluble extract	0.99%
Nonvolatile ether-soluble extract	0.94%

Crude Fiber.—Two samples of the drug yielded an average of 36.32% of crude fiber by the U. S. P. XI method (4).

Alkaloids.—The immiscible solvent method of the U. S. P. XI was used as a basis for the qualitative test for alkaloids. Five portions of the prepared drug were extracted separately, one with 50% alcohol and water, another with chloroform, a third with ether, a fourth with petroleum ether, and a fifth and last part with toluene as the solvents, respectively. The extracts were clarified by filtration, concentrated with gentle heat, and purified by extraction with successive portions of hydrochloric acid T. S., ammonium hydroxide T. S., and chloroform. This purification process was repeated five times and the final acid extract (containing any alkaloidal salt) in each case neutralized with sodium carbonate and tested for the presence of alkaloids by the common precipitation tests.

Mayer's reagent, saturated solution of picric acid, 0.1 *N* iodine, and 10% tannic acid solution all failed to give a positive test or reaction.

Nitrogen from Alkaloidal Matter.—The drug was extracted in the customary manner with acidified alcohol. The extract was purified by evaporation at reduced pressure and treated with absolute alcohol. The filtrate was subjected to the usual immiscible solvent extraction of alkaloidal matter. The final chloroform solution of any alkaloidal matter was allowed to evaporate to dryness at room temperature and this residue subjected to the usual test for nitrogen using metallic sodium.

No characteristic Prussian blue color or precipitate was obtained which indicated that nitrogen-containing substances such as alkaloids were absent.

Qualitative Analysis for Sugars.—A water extract and an 80% alcohol extract of the drug were prepared. The aqueous extract was treated with lead acetate solution, filtered, and the excess lead removed by using hydrogen sulfide. It was then freed from the latter by washing and finally repeatedly filtered until clear. The alcohol extract was cleared with lead acetate and the excess of lead removed by precipitation with potassium oxalate.

The filtered, clear, aqueous extract gave positive Molisch, Benedict's qualitative, Barfoed's, and osazone tests and negative Seliwanoff, mucic acid, and Tollen's orcinol tests. The prepared alcohol extract gave positive Molisch, Benedict's qualitative, and osazone tests and the others were negative. These results, coupled with the crystallization of the osazones only on cooling indicate the presence of maltose in the extracts. Microscopically the yellow rosette-like crystals in the form of broad lamina with pointed apices are characteristic of maltosazone.

Quantitative Analysis of Sugars.—An aliquot of a 90% alcohol extract of the drug was subjected to the method for the quantitative determination of sugars of the Official Agricultural Chemists (6).

The extract was prepared by evaporating the alcohol and adding some water to the soft, gummy residue. Lead acetate solution was added, the excess lead removed with potassium oxalate, and the mixture diluted with water to a definite volume and then filtered. This filtrate analyzed by the Munson and Walker method (7) for sugars indicated total sugar calculated as maltose, dextrose, and invert sugar of 0.80, 0.43, and 0.45%, respectively.

An aliquot of the same filtrate was heated with hydrochloric acid on a water bath for one-half hour, neutralized with sodium bicarbonate, and then diluted to volume. Invert sugar in this was determined quantitatively by the Munson and Walker method (7). The invert sugar in this prepared solution calculated as maltose, dextrose, and invert sugar equaled 1.55, 0.84, and 0.88%, respectively.

Glycoside.—For extracting the glycoside a modification of a process by Rosenthaler (8) was employed.

Some calcium carbonate was added to 95% alcohol and 250 Gm. of the coarsely ground drug was slowly added to the alcohol mixture kept boiling on a water bath. After all the drug had been added the boiling was continued for two hours under a reflux condenser. The mixture was filtered while hot and the drug refluxed again with hot alcohol. The combined hot alcohol extracts were allowed to cool and a grayish-white mucilaginous substance separated and was filtered out and preserved. The clear filtrate was evaporated to dryness at 20° and 20 mm. pressure. The dry residue was taken up with hot water and neutral lead acetate was used as a precipitant. After filtration the excess of lead was removed with hydrogen sulfide and the lead-free filtrate evaporated to a syrup at 18° and 20 mm. pressure.

This syrup was extracted with a hot mixture of equal parts of alcohol and ethyl acetate. Enough hot ethyl acetate was added slowly until two layers formed which were separated. The ethyl acetate layer was allowed to evaporate spontaneously in a beaker protected from dust. After about one week numerous crystal aggregates formed.

The aqueous syrup-like layer yielded some crystals after refluxing with ethyl acetate for two hours, the ethyl acetate layer separated, and crystallized as above. No additional crystals could be obtained, nor could any crystals be obtained from any of the other fractions mentioned.

The crystal products were recrystallized from a hot mixture of equal parts of alcohol and ethyl acetate. It was found that either alcohol or ethyl acetate alone could be used as the solvent for recrystallization. The individual crystals of the white bundle-like aggregates were needle-shaped and often grouped into "feather-duster" or "fan-like" bundles resembling shocks of grain. The dried crystals melted with decomposition at 160°.

Pure maltose (Merck) recrystallized in the same way resulted in identical crystal forms which melted with decomposition at 161.50°.

Nitrogen Determination.—The Kjeldahl method, modified to include nitrogen of nitrates, was used (5). The substitution of 1.0 Gm. of anhydrous copper sulfate for the 0.7 Gm. of mercuric oxide called for seemed to improve the method, which gave duplicate results of 0.68% of nitrogen. Calculated from the total nitrogen by the formula N (% of nitrogen) × 6.25 = % of protein. The result was 4.25%.

Volatile Oil.—The method of the U. S. P. XII (9) was used to determine the volatile oil in the drug, and yielded 0.45%. The volatile oil was lighter than water, had an aromatic dill to camphoraceous odor, and was colorless at first, gradually becoming brilliant light yellow, probably because of oxidation during the process of distillation. Index of refraction for the colorless fraction at 12° was 1.4720, of the mixture of colorless and colored fractions at 25° was 1.4712. The specific gravity of the colorless oil, determined by the use of a capillary Sprengel tube, was 0.9679.

The camphoraceous or borneol-like odor of the oil suggested an investigation for their presence. No phenylurethane derivative of the oil (10) could be prepared, hence borneol or other alcohols were considered to be absent. A semicarbazone (11) was prepared, which suggested an aldehyde and/or ketone. This semicarbazone melted at 193.5° with decomposition; that of camphor melts at 238°.

Androgenic and Estrogenic Principles.—In an attempt to isolate steroidal matter from *Chrysactinia Mexicana*, a procedure for the separation of the androgenic and estrogenic components in human urine devised by Pincus (12) and modified so as to include plant drug extracts by Costello (13) was applied.

A kilogram of the coarsely ground drug was extracted in a Soxhlet apparatus with 95% alcohol, the alcohol removed from the extract at 18° and 20 mm. pressure, thus yielding a black resinous mass. This residue was hydrolyzed by boiling it with 3 L. of 12% hydrochloric acid for seven minutes. The filtrate from this hydrolysis was discarded and the hydrolyzed residue was completely extracted with peroxide-free absolute ether. This ether extract was washed with distilled water and the washings separated and discarded. The ether extract was finally washed with several 10–15 cc. volumes of saturated sodium carbonate solution which removed acidic material, pigments, and the like. The acid-free ether extract and the sodium carbonate washings were separated, filtered, and retained for individual tests.

The acid-free ether extract was then extracted with 2 *N* sodium hydroxide and both solutions separated and filtered. The ether solution now represented the total ketosteroids or androgenic fraction. The alkaline solution represented the total phenolic or estrogenic fraction.

The ether solution, the androgenic fraction, was washed free of alkali with distilled water, and evaporated to dryness at 15° and 20 mm. pressure. A dark green, oleoresinous material weighing 2.5 Gm. was obtained. All of this material was dissolved in 100 cc. of 95% alcohol and filtered until clear in preparation for colorimetric assay by the Pincus modification of the Zimmerman reaction (14).

Aliquot portions of this total androgenic fraction were treated with 0.2 cc. of a 2% solution of *m*-dinitrobenzene in alcohol, followed by 0.2 cc. of 2.5 *N* aqueous potassium hydroxide and mixed well. This mixture was then placed in the dark at room temperature for forty-five minutes, and exactly three minutes before colorimetric measurement the prepared samples were diluted to 20 cc. with 60% alcohol. The measurements were made with a

Fisher Electrophotometer provided with a 510–520 mμ filter. Each aliquot was corrected for any color present by using a blank sample with the usual amount of potassium hydroxide but no *m*-dinitrobenzene solution. Solutions of dehydroisoandrosterone and of testosterone, which were available, were used as comparative standards. The aliquots of this total androgenic fraction treated with *m*-dinitrobenzene in the presence of alkali developed the characteristic violet-red color which became brownish orange after standing forty-five minutes at room temperature.

TABLE I.—TESTOSTERONE SOLUTION (100 γ/Cc.)

Volume Used, Cc.	Scale A	Scale B, %
0.1	2.1	95.4
0.3	2.75	94.0
0.5	3.6	92.0

TABLE II.—DEHYDROISOANDROSTERONE (100 γ/Cc.)

Volume Used, Cc.	Scale A[a]	Scale B,[a] %
0.05	3.6	94.4
0.1	4.0	91.25
0.25	5.8	87.5
0.3	5.9	87.25
0.5	6.5	86.2
0.6	6.4	86.4

[a] NOTE: Scale A is a logarithmic calculation, and Scale B is the percentage of light transmission.

TABLE III.—UNKNOWN (ANDROGENIC FRACTION) (2.5 Gm./100 Cc.)

Volume Used, Cc.	Scale A	Scale B, %
0.0017	2.6	93.6
0.0051	3.9	91.5
0.0085	4.2	90.9
0.043	20.8	62.0
0.1	39.9	40.0
0.129	52.3	30.0
0.215	61.1	24.5
0.3	70.0	20.0
0.5	96.0	1.0

The material tested was not a pure substance so the results obtained do not positively identify the androgenic material but the characteristic color reaction obtained is highly suggestive of 3 and/or 17-ketosteroids. Zimmerman (15) ascribes the qualitative color reaction of ketosterols with *m*-dinitrobenzene, sensitivity 1:100,000, to a violet-red decomposable dye, which in the case of relatively stable compounds is slowly converted into a second brownish orange dye. He assumed that the violet dye first formed was an addition compound of the quinol-nitro acid with the ketosterol held together through secondary valence bonds, and that the C—C bond becomes active only in the second brownish orange compound. Zimmerman further stated that in spite of the presence of an active methylene group, 6-keto, 7-keto, and 12-keto compounds do not give the reaction, and the presence of the functional groups in the polar positions (3- or 17-) is necessary for the development of the color reaction, just as the same configuration is necessary for physiological activity. Sterols having no keto group fail to give the color reaction. Zimmerman tabulated other substances having active methylene groups which give the reaction.

The alkaline solution, representing the total phenolic or estrogenic fraction, was brought to a *p*H of 2.5 with concentrated hydrochloric acid, filtered, and the filtrate extracted with ether. The ether extract was evaporated to dryness at 15° and 20 mm. pressure and 0.5 Gm. of residue obtained. A sighting experiment, employing biological methods of assay upon ovariectomized rats, was performed on this estrogenic fraction. The results were indefinite, but indicative of some estrogenic activity. This phenolic or estrogenic fraction should be combined with the sodium carbonate washings in order to obtain a true picture of the total estrogenic activity.

SUMMARY

1. The aerial portion of the shrub *Chrysactinia Mexicana*, A. Gray, was subjected to proximate analysis and a series of extracts prepared.

2. Various qualitative tests were performed upon extracts of the prepared drug.

(*a*) Alkaloidal material was considered to be absent in the extracts tested.

(*b*) The sugar, maltose, was isolated from an extract of the drug.

(*c*) Forty-five hundredths of a per cent of volatile oil was obtained. This oil has a dill or camphor-like odor and showed the presence of a keto-like compound, the semicarbazone of which melted at 193.5° with decomposition.

3. Color reactions with *m*-dinitrobenzene were highly suggestive of the presence of 3-ketosteroid, 17-ketosteroid or of 3-17-diketosteroid in an extract.

4. Some estrogenic activity was displayed in a biological assay experiment upon ovariectomized rats by a fraction from an extract.

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Notes

A Note on the Purification of Dehydrocholic Acid*

BY JOSE ERDOS

THE inherent difficulties encountered in the purification of dehydrocholic acid are well known. It is relatively easy to prepare this compound with a melting point of between 230 and 236°. The elimination of traces of steroids and of other acids with structures similar to that of dehydrocholic acid, however, is difficult to accomplish. In this laboratory we have used for some time a simple method of purification by which it is possible to obtain dehydrocholic acid having a melting point of 238 to 239°.

METHOD

One hundred grams of dehydrocholic acid (m. p. 230 to 236°) was mixed thoroughly with 230 cc. of water, and to this mixture 10 Gm. of sodium hydroxide dissolved in 20 cc. of water was added. When the acid was completely dissolved, 5 Gm. of Celite was added, the mixture stirred for ten minutes, and then filtered. To the filtrate, sodium chloride (35 Gm. for each 100 cc.) was added with continuous stirring while the mixture was heated to 60°. It was then cooled to approximately 5° and kept at that temperature overnight.

The mixture was then filtered by suction and the sodium dehydrocholate thus obtained was dissolved in 1 liter of water, and after the addition of Celite (Supercell), filtered. The filtrate was diluted with another liter of water, and to it was added diluted hydrochloric acid (1 in 2) to produce a *p*H of between 2.5 and 2. The dehydrochloric acid thus precipitated was collected on a filter, washed with water until the washings were free from chloride, and finally dried. The melting point of this product was 238°. Yield: 35–40 Gm., 35–40%. By repeating the procedure described above, or by recrystallizing from propyl alcohol, the melting point of this dehydrocholic acid was increased to 239.5°.

* Received January 21, 1950, from the Laboratory for Organic Chemical Investigation of the National Polytechnic Institute, Mexico, D. F.

A Note on the Isolation of Ursolic Acid from *Verbena stricta*, Vent.*

By NYDIA M. KING,† ASIMA CHATTERJEE,‡ and LLOYD M. PARKS

IN THE isolation and purification of the glycoside, verbenalin, from *Verbena stricta*, Vent. (1), there was accumulated a considerable quantity of a light yellowish green amorphous substance which, because of its high melting point, insolubility in the usual organic solvents, color reactions and other properties, was suspected to be, or to contain, a triterpene compound. From this we have isolated ursolic acid and identified it by means of its chemical properties and derivatives.

EXPERIMENTAL

For the routine isolation of verbenalin, the dried powdered drug was extracted with ether in 2-Kg. portions in a Soxhlet apparatus. During extraction the crude glycoside deposited in the bottom of the flask and the supernatant liquid was decanted and used to extract a fresh portion of drug. For purification the crude verbenalin was dissolved in hot alcohol and kept overnight, when the container was filled with a slimy magma of gelatinous appearance. This was separated by filtration. Concentrating the filtrate and allowing it to stand yielded more of the slimy magma. Repetition of this procedure was used to remove most of the nonglycosidal material from the alcohol solution which, when finally concentrated to a small volume, yielded the crystalline glycoside. In this way there was accumulated approximately 30 Gm. of the amorphous so-called slimy matter which melted indefinitely over a range in the vicinity of 250°.

Ten grams of the amorphous substance was dissolved by long refluxing in 500 cc. of ether; to the cooled solution in a separatory funnel 5% aqueous NaOH was added, with the formation of a yellowish precipitate at the interface. The precipitate was removed by suction filter and the aqueous alkali layer was separated from the filtrate. The addition

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of more 5% alkali to the ether layer, followed by filtration, was continued until no more precipitate was obtained.

The dried precipitate, thus obtained, was dissolved by long refluxing in 900 cc. of absolute alcohol, the solution filtered, acidified to Congo red with dilute HCl, and poured into 1 L. of water, with the separation of a white flocculent precipitate. This precipitate was collected by suction, dried, dissolved in alcohol, and the alcohol solution decolorized with Norite. Upon standing there was deposited from the alcohol 4 Gm. of white amorphous solid which melted indefinitely over a range in the vicinity of 260°.

To remove lipoidal matter the 4 Gm. of amorphous solid was extracted by refluxing with three successive 100-cc. portions of Skellysolve A. The unextracted residue was dissolved in 5% aqueous-alcoholic KOH (3:7), the solution digested with Norite, and the filtrate acidified with dilute HCl. The resulting precipitate, after several recrystallizations from absolute alcohol, yielded needle-like crystals, m. p. 282°, which gave the typical color reaction of a triterpene hydroxy acid with the Lieberman-Burchard test and did not depress the melting point of an authentic sample of ursolic acid (m. p., 281–284°; mixed m. p., 282–284°).

Anal. Calcd. for $C_{30}H_{48}O_3$: C, 78.95; H, 10.53; equiv. wt., 456. Found:[1] C, 78.62; H, 10,64; equiv. wt., 456.1, 455.9. $[\alpha]^{27.5^\circ}_D = +65.6, +63.1$ (C = 2.406 in 1 *N* alcoholic KOH). An authentic sample of ursolic acid showed $[\alpha]^{27.5^\circ}_D = +61.7, +60.7$ (C = 3.26 in 1 *N* alcoholic KOH); literature reports $[\alpha]^{24.5^\circ}_D = +62.1, +62.6$ (2).

The acetate, m. p. 282°, and methyl ester, m. p. 167°, prepared from the ursolic acid which was isolated from *Verbena stricta*, did not depress the melting point of the corresponding derivative of an authentic sample of ursolic acid (m. p. of acetate, 286–288°; mixed m. p. 282–285°; m. p. of methyl ursolate, 167–168°; mixed m. p. 167°).

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[1] We are indebted to Professor A. L. Wilds, Chemistry Department, University of Wisconsin, for the combustion data.

Book Reviews

Surface-Active Quaternary Ammonium Germicides. By CARL A. LAWRENCE. Academic Press, Inc., New York, 1950. vii + 245 pp. 15.5 x 23.8 cm. Price $6.

In this book the author has provided a heretofore much needed review of the entire field of quaternary-ammonium surface-active germicides. The developments in this field have come, for the most part, during the past ten years. The author of this review was intimately identified with these developments and because of this he is eminently qualified to review and summarize the extensive literature relating to the quaternary ammonium compounds. He has accomplished his aim in a most satisfactory manner, and the book should prove to be of value to anyone interested from any viewpoint in these unique compounds which possess germicidal, surface-active, detergent, and wetting properties. The book is divided into 11 sections which include summaries on the chemistry and the physical properties of the quaternary ammonium compounds, biological considerations, and pharmacological and toxicological data.

Approximately half of the text is utilized in summarizing available information on the practical applications of the quaternary ammonium compounds to surgery, as general disinfectants, and to the various industries in which they are employed.

A final section consists of a table of surface-active agents and germicides in which the quaternary ammonium compounds presently available are listed by trade name, chemical name, and distributor or manufacturer. The book is well documented with 550 references and is provided with an author index. The subject index is well planned and quite adequate for the location of information on any one of the many ramifications of fields of interest involving the quaternary ammonium compounds.

In the introduction the author states: "The purpose of this monograph is to accumulate in the pages of one volume a review of the history, chemistry, biology, and application of the quaternary ammonium salts that have appeared in print to date." It is believed that anyone using the monograph will enthusiastically agree that the purpose stated by the author has been adequately achieved.

Chemical Constitution and Biological Activity. By W. A. SEXTON. D. Van Nostrand Company, New York, 1950. xxi + 412 pp. Illustrated. 15.5 x 25.5 cm. Price $10.

This book presents a more advanced view of the realtionship of chemical structure to biological activity than is generally included in books on pharmaceutical chemistry. It will be of particular interest to the graduate student and to the teacher, and

should hold considerable interest for workers in biochemistry and pharmacology as well as pharmaceutical chemistry.

It is encouraging to see that the author reviews some of the newer concepts of protein structure before introducing the subject of enzyme action and inhibition. He then presents a brief discussion of noncompetitive and competitive enzyme inhibitors. This could be made more effective perhaps if more of the mathematical treatment of the subject were included, but the explanations and definitions seem quite clear, and the material is well organized.

A chapter on physicochemical aspects of drug action reviews the various theories based on physical characteristics of drug molecules. The author discusses the biological effects which may be due to differences in bond energy, molecular size and shape, and stereochemical structure. Cell permeability and orientation of monomolecular layers are also considered briefly.

The section on effects of specific chemical group is especially interesting. In it are pointed out the effects that have been attributed to the addition of methyl, hydroxyl, thiol, and other groups to various types of molecules.

Several of the known classes of metabolites are then taken up in order with some information as to their biological function, the nature of their combinations, and some discussion of competitive analogues. Included in this group are the vitamins, the porphyrins, choline derivatives, quinones, and antibiotics. In later chapters are discussed hormones, antiprotozoal drugs, fungicides, bactericides, and plant growth regulators.

One chapter contains a discussion of cancer with respect to the dyes specific for cancerous tissue, the chemicals which have shown some promise in treatment, and the carcinogenic compounds.

This book is very well illustrated and documented.

Proceedings of the First National Conference on Cardiovascular Diseases. Published by the American Heart Association. International Press, New York, 1950. xix + 259 pp. 15 x 23 cm. Price $1.75.

Three years ago, the American Heart Association existed only as a body of physicians interested in cardiovascular diseases. The National Heart Institute of the Public Health Service, Federal Security Agency, had not been created by Congress.

Since 1947 the Association has emerged as a voluntary agency with thousands of lay participants and the Institute (established in 1948) has put federal resources into the effort to prevent and control heart disease. Although many fundamental questions about heart disease are still unanswered, we know enough today to offer solid reassurance and help to many of the estimated 9,000,000 people with cardiovascular disease. Unfortunately, many of these cases have not been discovered, and the full resources of science and the community have not been used to help many of those classified as cardiacs.

To attack these problems, Dr. John W. Ferree, the Association's Director of Public Health, proposed that a representative group of experts in all fields related to cardiovascular disease be brought together and asked to help map a program. This plan was quickly approved by the Association and the Institute and the two agencies agreed to sponsor a national conference.

The National Conference on Cardiovascular Diseases, held in Washington, January 18–20, 1950, brought together 196 distinguished professional and lay leaders to determine what we know about cardiovascular disease and how we can apply knowledge to prevent and cure it.

The conference was a decided success, and before the eleven committees left Washington, they wrote the reports on their separate committee deliberations. Since the committees were kept small, the Association decided to print a brief summary of the conference for distribution to those who could not be present.

The complete record of the conference is contained in this paper-bound book, which is divided into four parts: (1) Stenographic transcripts of the general meetings held on the first and third days; (2) papers and reports of the section on Technical Knowledge and Research; (3) reports of the section on Community Services and Facilities; and (4) reports of the section on Professional Education. Individual reports are signed with the author's name.

The book is well organized, unlike many conference reports, and is condensed enough so that it should prove useful as a means of quickly bringing the reader up-to-date on progress in the field of cardiovascular diseases.

An Introduction to the Study of Experimental Medicine. By CLAUDE BERNARD. Henry Schuman, Inc., New York, 1949. xix + 226 pp. 15.5 x 23.5 cm. Price $3.

This world-famous classic, first published in 1865, has again become available in English. Actually, it is more than a classic, it is a classic which is still read. It is in fact among the One Hundred Great Books which comprise the basic curriculum of St. John's College.

Claude Bernard is the founder of experimental medicine, i.e., the artificial production of disease by means of chemical and physical manipulation. His research in physiology, which is the foundation of scientific medicine and the most important part of biology, has immortalized his name. His important work included the study of the function of the pancreas gland and the glycogenic function of the liver which throws light on the cause of diabetes. In addition, Bernard is credited with the discovery of the vasomotor system.

For many readers the most interesting and valuable part of the *Introduction* will always be Bernard's descriptions of the successive steps of each of his principal discoveries and his consequent deduction of the way in which the mind of a scientist goes to work on a problem.

General physiology, of which Bernard is the founder, was destined to remain undeveloped until long after his death. Today, with the aid of a physical chemistry unknown to the contemporaries of Claude Bernard, it is fulfilling the promise which he alone could clearly see.

BOOKS RECEIVED

The Annual Reprint of the Reports of the Council on Pharmacy and Chemistry of the American Medical Association. J. B. Lippincott Company, Philadelphia, 1949. 231 pp. 12 x 20 cm. Price $2.

New and Nonofficial Remedies, 1950. The Council on Pharmacy and Chemistry, American Medical Association. J. B. Lippincott Company, Philadelphia, 1950. li + 800 pp. 13 x 19.5 cm. Price $3.

Essentials of Medicine. 16th ed. By CHARLES PHILLIPS EMERSON and JANE ELIZABETH TAYLOR. J. B. Lippincott Company, Philadelphia, 1950. xxii + 815 pp. Illustrated. 14 x 21 cm. Price $4.

Your Hair: Its Health, Beauty, and Growth. By HERMAN GOODMAN. Emerson Books, Inc., New York, 1950. xiii + 287 pp. Illustrated. 14 x 22.5 cm. Price $2.95.

Physico-Chemical Constants of Pure Organic Compounds. By J. TIMMERMANS. Elsevier Publishing Company, Inc., New York, 1950. viii + 693 pp. 17 x 24.5 cm. Price $12.50.

Cell Physiology and Pharmacology. By J. F. DANIELLI. Elsevier Publishing Company, Inc., New York, 1950. viii + 156 pp. Illustrated. 15.5 x 23.5 cm. Price $3.

The Condensed Chemical Dictionary. 4th ed. FRANCIS M. TURNER, Editorial Director. Revised and enlarged by Arthur and Elizabeth Rose. Reinhold Publishing Corporation, New York, 1950. xxix + 726 pp. 16 x 23.5 cm. Price $10.

The Biochemistry of B Vitamins. By ROGER J. WILLIAMS, ROBERT E. EAKIN, ERNEST BEERSTECHER, Jr., and WILLIAM SHIVE. Reinhold Publishing Corporation, New York, 1950. x + 741 pp. 15.5 x 23.5 cm. Price $10.

1951 Iodine Research Award Nominations Requested

Nominations are now being received by the AMERICAN PHARMACEUTICAL ASSOCIATION for the 1951 Chilean Iodine Educational Bureau Award recognizing outstanding research in the chemistry and pharmacy of iodine and its compounds as applied in pharmacy or medicine. Any member of the ASSOCIATION may propose a nominee by submitting eight copies of each of the publications to be considered in the competition, a biographical sketch of the nominee including date of birth, and a list of his publications. Eight copies of the nomination must be submitted to Robert P. Fischelis, Secretary of the AMERICAN PHARMACEUTICAL ASSOCIATION, 2215 Constitution Ave., N. W., Washington 7, D. C. To be eligible for the 1951 Award, nominations must be received on or before January 1, 1951.

A nominee must be a resident of the United States or Canada. He must have accomplished outstanding research in the chemistry or pharmacy of iodine and its compounds as applied in pharmacy or medicine.

During the period covered by the nomination the nominee shall have been actively engaged in, shall have completed, or shall have published a report upon the line of investigation for which the award is made. During the period of two years prior to the date of nomination, the nominee shall not have been engaged in research under the sponsorship of the Chilean Iodine Educational Bureau, Inc.

The award consists of $1000 and a diploma setting forth the reasons for selection of the recipient. It may be presented annually at the annual meeting of the ASSOCIATION.

The recipient will deliver a paper or lecture upon the subject of his scientific work at the meeting at which the award is conferred. His paper, or address, will then be published in the JOURNAL OF THE AMERICAN PHARMACEUTICAL ASSOCIATION. In addition to the sum of the award, the recipient will receive an allowance of not more than $250 to defray his expenses in attending the meeting.

The recipient will be selected by an award committee which is appointed by the chairman of the ASSOCIATION's Council. The present committee includes Justin L. Powers, chairman; Louis Gershenfeld, Charles O. Wilson, Harvey B. Haag, Heber W. Youngken, John C. Krantz, and Frank O. Taylor.

The award is now in its fourth year. The recipient of the first award was Dr. William T. Salter, Yale University pharmacologist and the second recipient was Dr. George M. Curtis, Chairman of the Department of Surgical Research and Professor of Surgery at Ohio State University.

Dr. C. P. Leblond, Professor of Anatomy, McGill University, Montreal, Canada, will receive the third award and will give the 1951 Iodine Award lecture.

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